

**CELL ADHESION OF HUMAN HAEMATOPOIETIC PROGENITORS.**

**Development of Assay Techniques.**

**MARC LEIGHTON TURNER.**

**DOCTOR OF PHILOSOPHY**

**UNIVERSITY OF EDINBURGH 1995**



## **DEDICATION.**

To the memory of my Mother and Grandmother.

To my wife with love.

To the future of our unborn child.



## **DECLARATION.**

I hereby declare that this thesis has been composed solely by myself and that the work described herein was my own except for the contributions of others indicated.

Marc L Turner

17th July 1995.

## ABSTRACT.

During bone marrow transplantation a conditioning regimen ablates endogenous haematopoiesis. This is reconstituted by intravenous infusion of haematopoietic progenitors (HPC) which home to and engraft the bone marrow, and are responsible for long-term support of the haematopoietic and lymphoid systems. HPC are present at low levels in the peripheral blood of normal individuals, but markedly raised levels are found under a variety of circumstances which permit HPC harvest and storage for future transplantation.

Elucidation of the mechanisms underlying the reciprocal phenomena of HPC homing and mobilisation is the objective of this thesis, and may allow the development of novel approaches to mobilisation regimens and / or *ex vivo* HPC manipulation.

Accurate and reproducible assays for quantitative and qualitative studies of HPC were established. These included an immunocytometry based assay of CD34 antigen expression, dual colour immunocytometry studies of cell adhesion molecule, lineage and activation marker expression by HPC derived from different sources, and three colour immunocytometry of adhesion molecule expression within HPC subsets. A <sup>51</sup>chromium-labelling technique was developed as a functional assay with which to examine the adhesion of haematopoietic cell lines to extracellular matrix components, stromal and endothelial tissues in culture. A variety of techniques for adhesion blockade were explored. A protocol for high-purity enrichment of HPC was developed, and the feasibility of applying the <sup>51</sup>chromium adhesion assay to these cells was examined.

CD34 immunocytometry was confirmed as a valid method for defining HPC populations. Marrow HPC were found to express nine adhesion molecules, two of which were reduced by circulating cells. HPC derived from different sources showed variation in lineage and activation marker expression, and HPC subsets displayed differences in adhesion molecule expression. Haematopoietic cell lines adhered to fibronectin and thrombospondin, but not to other extracellular matrix components. Blockade of fibronectin adhesion was effected by divalent cation

chelation, synthetic peptides and chondroitinase ABC. Cell line adhesion to stromal and endothelial tissue cultures was demonstrated, but highly-enriched HPC labelled poorly with  $^{51}$ chromium.

These studies have contributed to a better understanding of the pattern of cell adhesion molecule expression by bone marrow and circulating HPC, and the functional role of these molecules in HPC homing and mobilisation.

## **CONTENTS.**

<b>DEDICATION</b>	<i>p 2</i>
<b>DECLARATION</b>	<i>p 3</i>
<b>ABSTRACT</b>	<i>p 4</i>
<b>CONTENTS</b>	<i>p 6</i>
<b>LIST OF ABBREVIATIONS</b>	<i>p 10</i>
<b>LIST OF TABLES</b>	<i>p 12</i>
<b>LIST OF FIGURES</b>	<i>p 18</i>

### **CHAPTER 1. INTRODUCTION. *p 21***

- 1.1 Historical Background.**
- 1.2 Haematopoietic Stem and Progenitor Cells.**
- 1.3 Regulation of Haematopoiesis.**
- 1.4 Haematopoietic Progenitor Transplantation.**
- 1.5 Cell Adhesion and Position.**
- 1.6 Haematopoietic Progenitor Cell Adhesion and Objectives of the Study.**

### **CHAPTER 2. QUANTITATIVE DETERMINATION OF HAEMATOPOIETIC PROGENITORS. *p 63***

- 2.1 Introduction.**
- 2.2 General Methodology.**
- 2.3 Standard methodology for the CFU-GM Assay.**
- 2.4 Optimisation of CD34 Immunocytometry.**
- 2.5 Determination of Resolution Limits of Immunocytometry.**
- 2.6 Evaluation of Different Approaches to Data Analysis.**
- 2.7 Study of the Overall Accuracy of CD34 Quantitation.**
- 2.8 Establishment of a Correlation between CD34 and CFU-GM Assays.**
- 2.9 Discussion.**

**CHAPTER 3. CHARACTERISATION OF HAEMATPOIETIC  
PROGENITOR PHENOTYPE.**

*p 114*

**3.1 Introduction.**

**3.2 A Comparative Study of Haematopoietic Progenitor Cell Adhesion  
Molecule Expression.**

**3.3 A Comparative Study of Haematopoietic Progenitor Expression of  
Activation and Lineage Markers.**

**3.4 Characterisation of Cell Adhesion Molecule, Lineage and Activation  
Marker Expression by Haematopoietic Cell Lines.**

**3.5 Development of Three-Colour Immunocytometry to Study Cell Adhesion  
Molecule Expression within Haematopoietic Progenitor Subsets.**

**3.7 Discussion.**

**CHAPTER 4. DEVELOPMENT OF A <sup>51</sup>CHROMIUM ADHESION ASSAY  
TO STUDY FUNCTIONAL ADHESION OF HAEMATPOIETIC CELL  
LINES.**

*p 176*

**4.1 Introduction.**

**4.2 Optimisation of <sup>51</sup>Chromium Radiolabelling.**

**4.3 Optimal Coating of the Carrier with Substrate.**

**4.4 Standardisation of the Adhesion Assay.**

**4.5 Discussion and Formulation of Results.**

**CHAPTER 5. APPLICATION OF THE <sup>51</sup>CHROMIUM ADHESION ASSAY  
TO THE STUDY OF HUMAN HAEMATOPOIETIC CELL LINE  
ADHESION TO NON- CELLULAR SUBSTRATES. p 216**

**5.1 Introduction.**

**5.2 Study of the Adhesion of Human Haematopoietic Cell Lines to  
Extracellular Matrix Components.**

**5.3 Effects of Activating Agents on KG1a adhesion to Extracellular Matrix  
Components.**

**5.4 Studies of KG1a Binding to Fibronectin.**

**5.5 Discussion.**

**CHAPTER 6. APPLICATION OF <sup>51</sup>CHROMIUM ADHESION ASSAY TO  
THE STUDY OF CELLULAR SUBSTRATES AND TO ENRICHED  
POPULATIONS OF HAEMATOPOIETIC PROGENITORS. p 245**

**6.1 Introduction.**

**6.2 Preparation of *in vitro* Tissue Cultures.**

**6.3 Preparatory Experiments to Study the Adhesion of KG1a to *in vitro* Tissue  
Cultures.**

**6.4 Comparative Adhesion of Haematopoietic Cell Lines to M210-B4.**

**6.5 Blockade of KG1a Adhesion to M210-B4.**

**6.6 Development of a Haematopoietic Progenitor Enrichment Protocol.**

**6.7 Application of the <sup>51</sup>Cr Adhesion Assay to Highly Enriched Haematopoietic  
Progenitors.**

**6.8 Discussion.**

**CHAPTER 7. GENERAL DISCUSSION.** *p 290*

**7.1 Definition and Analysis of the Haematopoietic Progenitor Population.**

**7.2 Phenotypic Studies on Haematopoietic Progenitors.**

**7.3 Functional Studies of Haematopoietic Cell Line Adhesion.**

**7.4 Functional Assay of Haematopoietic Progenitor Adhesion.**

**7.5 The Limitations of Current Adhesion Assays.**

**7.6 Potential Clinical Applications.**

**7.7 Concluding Remarks.**

**BIBLIOGRAPHY.** *p 316*

**APPENDICES.** *p 356*

**Appendix 1. Materials and Suppliers.**

**Appendix 2. Preparation of Solutions and Media.**

**Appendix 3. Additional Tables.**

**Appendix 4. Human Bone Marrow Endothelial Cultures.**

**Appendix 5. Publications.**

**ACKNOWLEDGEMENTS** *p 389*

## LIST OF ABBREVIATIONS.

BM	bone marrow
BSA	bovine serum albumin
CAM	cell adhesion molecule
CB	umbilical cord blood
CD	cluster designation
CD34% <sup>+</sup>	overall percentage CD34 positive cells
CD34% <sup>c</sup>	CD34 positive cells as a percentage of CD45 <sup>+</sup> leucocytes
CD34 <sup>n</sup>	absolute number of CD34 positive cells
CE	cloning efficiency
CFU-GM	colony forming unit - granulocyte-monocyte
<sup>51</sup> Cr	chromium 51 (sodium chromate)
cv	coefficient of variation
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
FACS	fluorescence activated cell scanner
FACSort	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL-1	fluorescence channel 1
FL-2	fluorescence channel 2
FL-3	fluorescence channel 3
FSC	forward light scatter
GAM	goat anti-mouse
HBSS	Hanks' balanced salts solution
HPC	haematopoietic progenitor cells
IMDM	Iscoe's modified Dulbecco's medium
LI	labelling index
Lin	lineage / activation marker
LP	leucapheresis product



LTBMC	long-term bone marrow culture
mAb	monoclonal antibody
mfi	mean fluorescence intensity
MNC	mononuclear cells
n	number (of cells or experiments)
ns	not significant
<i>p</i>	pagination
<i>p</i>	probability (Student's <i>t</i> test)
PB	peripheral blood
PB <sup>m</sup>	peripheral blood (mobilised)
PBS	phosphate buffered saline
%fl <sup>+</sup>	percentage fluorescence positive
pFn	plasma fibronectin
PMA	phorbol myristate acetate
<i>r</i>	correlation coefficient
RAM	rat anti-mouse
RGDS	arginine-glycine aspartic acid-serine
RGES	arginine-glycine-glutamic acid-serine
rhG-CSF	recombinant human granulocyte colony stimulating factor
RI	resolution index
RPE	R-phycoerythrin
SAM	sheep anti-mouse
SD	standard deviation
SSC	side light scatter
tFn	tissue fibronectin

*Standard SI units and abbreviations are used throughout.*

## **LIST OF TABLES.**

**Table 1.1 Regulatory Cytokines. *p* 38.**

**Table 1.2 Endocrine Factors Contributing to Haematopoietic Regulation. *p* 38.**

**Table 1.3 Haematopoietic Cytokine Receptors. *p* 40.**

**Table 1.4 Clinical Indications for Bone Marrow Transplantation. *p* 42.**

**Table 1.5 Conditions giving rise to Elevation of Circulating Haematopoietic Progenitors. *p* 44.**

**Table 1.6 Candidate Diseases for Correction by Gene Transfer into Haematopoietic Progenitors. *p* 49.**

**Table 1.7 Families of Cell Adhesion Molecules. *p* 51.**

**Table 1.8 Members of the Immunoglobulin Gene Superfamily. *p* 53.**

**Table 1.9 Members of the Integrin Family. *p* 54.**

**Table 1.10 Members of the Selectin Family. *p* 56.**

**Table 1.11 Other Cell Adhesion Molecules. *p* 57.**

**Table 2.1 Human Haematopoietic Cell Lines Studied. *p* 67.**

**Table 2.2 Titration of 8G12-RPE on KG1a. *p* 78.**

**Table 2.3 Test Panels for CD34 Quantitation. *p* 81.**

**Table 2.4 Effects of Erythrocyte Lysis on Physical and Fluorescence Characteristics of CD34 and CD45-Labelled Cells. *p* 83.**

**Table 2.5 Effects of Paraformaldehyde Storage on Physical and Fluorescence Characteristics of CD34 and CD45-Labelled Cells. *p* 85.**

**Table 2.6 Flow Cytometry Settings for Single and Dual Colour Immunocytometry on Leucocytes. *p* 86.**

**Table 2.7 Evaluation of anti-CD34 (FITC) Monoclonal Antibodies on KG1a. *p* 90**

**Table 2.8 Evaluation of anti-CD34 (RPE) Monoclonal Antibodies on KG1a. *p* 91.**

**Table 2.9 Evaluation of Negative Control Antibodies on KG1a. *p* 92.**

**Table 2.10 Synopsis of the Single Colour Immunocytometry Labelling Protocol. *p* 95.**

**Table 2.11 Serial Dilution of KG1a in Peripheral Blood-Mononuclear Cells.**  
*p 97.*

**Table 3.1 Cell Adhesion Molecules Studied.** *p 117.*

**Table 3.2. Dual Immunofluorescence Labelling During the Cell Adhesion Molecule Study.** *p 119.*

**Table 3.3. Test Panel for the Cell Adhesion Molecule Study.** *p 120.*

**Table 3.4 Comparative Statistics on CD34<sup>+</sup> Cell Adhesion Molecule Expression between Bone Marrow, Mobilised Peripheral Blood and Cord Blood.** *p 126.*

**Table 3.5 Effects of Incubation with and without Mouse Serum.** *p 128.*

**Table 3.6 Effect of Mouse Serum on CD45 SAM-RPE Labelling of Peripheral Blood Mononuclear Cells.** *p 128.*

**Table 3.7 Marker Channel for Each of the Sources.** *p 129.*

**Table 3.8 Lineage and Activation Markers Studied.** *p 131.*

**Table 3.9 Modified Dual Immunofluorescence Labelling Protocol used for the Analysis of Lineage Marker Expression.** *p 133.*

**Table 3.10 Test System for Lineage Marker Study.** *p 134.*

**Table 3.11 Comparative Statistics on Lineage Marker Expression between Bone Marrow, Mobilised Peripheral Blood and Matched Leucapheresis Product and Cord Blood.** *p 139.*

**Table 3.12 Flow Cytometer Settings for Cell Line Studies.** *p 142.*

**Table 3.13 Three Colour Immunofluorescence Labelling Protocol.** *p 149.*

**Table 3.14 Flow Cytometer Settings for Three Colour Immunocytometry.** *p 160*

**Table 3.15 Test Panel for the Three Colour Immunocytometry Study.** *p 164.*

**Table 3.16 Percentage Positive CAM Fluorescence within CD34<sup>+</sup> Lin<sup>+</sup> and Lin<sup>-</sup> Subsets.** *p 167.*

**Figure 3.17 Mean Fluorescence Intensity of CAM Expression within CD34<sup>+</sup> Lin<sup>+</sup> and Lin<sup>-</sup> Subsets.** *p 168.*

**Table 3.18 Statistical Analysis of CAM Expression within CD34<sup>+</sup> Lin<sup>+</sup> and Lin<sup>-</sup> Subsets.** *p 169.*

Table 4.1 Resolution Limits of the Gamma Counter using Serial Dilutions of $^{51}\text{Chromium}$ .	p 182.
Table 4.2 Effect of $^{51}\text{Chromium}$ Concentration on Labelling Index and Cell Viability.	p 184.
Table 4.3 Effect of Serial Washing on the Supernatant Contamination.	p 185.
Table 4.4 Alterations in Cell Viability and Supernatant Contamination over the Expected Experimental Period.	p 187.
Table 4.5 Correlation of $^{51}\text{Chromium}$ -Labelling with Actual Cell Numbers.	p 188.
Table 4.6 Effects of Coating Conditions on KG1a Adherence to Fibronectin and Albumin Coated Tissue Culture Wells.	p 193.
Table 4.7 Binding of KG1a to Fibronectin under Varying Coating Concentrations.	p 194.
Table 4.8 Effects of Assay Medium on KG1a Adhesion to Various Substrates.	p 195.
Table 4.9 Effects of Pre-Incubation with 1% Denatured BSA and Number of Washes on KG1a Adherence to Fibronectin and Albumin.	p 198.
Table 4.10 Effects of Shaking or Not-Shaking the Plate between Washes.	p 199.
Table 4.11 Temperature Dependence of the Assay System.	p 199.
Table 4.12 Temporal Dependence of the Assay System.	p 200.
Table 4.13 Effects of Variation in the Depth of Suspension Medium on KG1a Binding to Fibronectin.	p 203.
Table 4.14 Effect of Centrifugation on Overall Cell Adhesion.	p 204.
Table 4.15 Concentration Dependence of the Assay System.	p 205.
Table 4.16 Effects of Replating Non-Adherent Cells.	p 207.
Table 4.17 Stability of the Labelling Indices.	p 208.
Table 4.18 Subanalysis of Fibronectin and dBSA Adhesion.	p 208.
Table 4.19 Background Adhesion of KG1a to Different Batches of BSA.	p 209.
Table 4.20 Summary of the Standardised Cell Adhesion Assay.	p 214.
Table 5.1 Extracellular Matrix Components Studied.	p 218.

Table 5.2 <sup>51</sup> Cr Labelling of Haematopoietic Cell Lines.	p 220.
Table 5.3 Adhesion of KG1a to Extracellular Matrix Components.	p 221.
Table 5.4 Adhesion of K562 to Extracellular Matrix Components.	p 222.
Table 5.5 Adhesion of HL60 to Extracellular Matrix Components.	p 223.
Table 5.6 Adhesion of NALM-6 to Extracellular Matrix Components.	p 224.
Table 5.7 Adhesion of CEM to Extracellular Matrix Components.	p 225.
Table 5.8 Effects of PMA on KG1a Adhesion to Extracellular Matrix Components.	p 228.
Table 5.9 Effects of A23187 on KG1a Adhesion to Extracellular Matrix Components.	p 230.
Table 5.10 Comparative Adhesion of KG1a to Plasma and Tissue Fibronectin.	p 235.
Table 5.11 Effects of Metabolic Inhibitors on KG1a Adhesion to Plasma Fibronectin.	p 236.
Table 5.12 Blockade of KG1a Adhesion to Fibronectin by RGD Containing Peptides.	p 238.
Table 5.13 KG1a Adhesion to Fibronectin in the Presence of Monoclonal Antibodies.	p 240.
Table 5.14 Comparative Effects of Enzymatic Digestion of Proteoglycans on KG1a Adhesion to Fibronectin.	p 242.
Table 6.1 Cr <sup>51</sup> Uptake by the Adherent Stromal Layer.	p 258.
Table 6.2 Comparative Adhesion of KG1a to the M210-B4 Cell Line and to Human Bone Marrow Fibroblast and Stromal Cultures.	p 259.
Table 6.3 Statistical Analysis of KG1a Adhesion to Various Combinations of Cellular Substrate.	p 260.
Table 6.4 Comparative Adhesion of Haematopoietic Cell Lines to M210-B4.	p 261.
Table 6.5 Blockade of KG1a-M210-B4 Adhesion by Divalent Cation Chelation and RGDS Peptides.	p 263.

**Table 6.6 Blockade of KG1a-M210B4 Adhesion by Monoclonal Antibodies.**

***p 265.***

**Table 6.7 Blockade of KG1a-M210-B4 Adhesion by Enzymes. *p 266.***

**Table 6.8 Haematopoietic Progenitor Enrichment. *p 268.***

**Table 6.9 Pilot Cell-Sort of  $10^8$  Cells from Leucapheresis Product. *p 272.***

**Table 6.10 Results of Preparatory Separation of Bone Marrow Samples over Ficoll-Hypaque. *p 272.***

**Table 6.11 Results of Experiments Removing Dynal-Beads from KG1a using the Detachabead System. *p 277.***

**Table 6.12 Immunofluorescence Labelling Protocol for *MACS* and FACSsorting. *p 278.***

**Table 6.13 Results of the Effects of *MACS* Separation on Leucapheresis Product. *p 281.***

**Table 6.14 Results of Sequential *MACS* and FACSsort Enrichment of HPC on the basis of CD34 Positivity. *p 282.***

**Table 6.15  $\text{Cr}^{51}$  Labelling of Peripheral Blood Lymphocytes. *p 285.***

**Table 6.16  $\text{Cr}^{51}$  Labelling of Enriched Haematopoietic Progenitors. *p 287.***

**Table A1 Overall Accuracy of CD34<sup>+</sup> Quantitation. *p 364.***

**Table A2 Correlation between CFU-GM and CD34<sup>+</sup> Cells in 14 Leucapheresis Product Samples. *p 365.***

**Table A3 Analysis of CD34<sup>+</sup> Counts and CFU-GM in Normal Individuals. *p 366.***

**Table A4 Percentage Positivity Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood. *p 367.***

**Table A5 Mean Fluorescence Intensity of Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood. *p 369.***

**Table A6 Percentage Positive Lineage Marker Expression within the CD34<sup>+</sup> Populations of Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood. *p 371.***

**Table A7 Mean Fluorescence Intensity of Lineage Marker Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood. *p* 372.**

**Table A.8 Lineage, Activation and Cell Adhesion Markers Expressed by KG1a. *p* 373.**

**Table A.9 Lineage, Activation and Cell Adhesion Markers Expressed by K562. *p* 375.**

**Table A.10 Lineage, Activation and Cell Adhesion Markers Expressed by HL60. *p* 377.**

**Table A.11 Lineage, Activation and Cell Adhesion Markers Expressed by NALM-6. *p* 379.**

**Table A.12 Lineage, Activation and Cell Adhesion Markers Expressed by CEM. *p* 381.**

**Table A.13 Calibration using Microbeads. *p* 383.**

**Table A.14 Adhesion of Human Haematopoietic Cell Lines to Human Umbilical Vein and Bone Marrow Endothelial Cultures. *p* 386.**



## **LIST OF FIGURES.**

**Figure 1.1 The Stem Cell Model of Haematopoiesis. *p* 24.**

**Figure 2.1 CFU-GM Colony Assays. *p* 70.**

**Figure 2.2 Design of the Flow Cytometer. *p* 73.**

**Figure 2.3 FSC / SSC Characteristics of Mature Cell Populations. *p* 74.**

**Figure 2.4 Negative and Positive Controls and Compensation. *p* 77.**

**Figure 2.5 Backgating on the CD45<sup>+</sup> Population to Define Acquisition Gates for CD34<sup>+</sup> Enumeration. *p* 87.**

**Figure 2.6 CD34 -Labelling of KG1a With and Without Backgating. *p* 89.**

**Figure 2.7 Comparative Approaches to CD34 Enumeration. *p* 98.**

**Figure 2.8 CD45<sup>+</sup> Fluorescence of Various Populations of Cells. *p* 100.**

**Figure 2.9 Overall Accuracy of CD34<sup>+</sup> Quantitation. *p* 102.**

**Figure 2.10 Correlation between CFU-GM and CD34<sup>+</sup> Cells in 14 Leucapheresis Samples. *p* 104.**

**Figure 2.11 Methods of Discrimination of Positive and Negative Populations. *p* 110.**

**Figure 3.1 Dual Immunocytometry Studies of Cell Adhesion Molecule Expression by Haematopoietic Progenitors. *p* 123.**

**Figure 3.2 Percentage Positivity Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood. *p* 124.**

**Figure 3.3 Mean Fluorescence Intensity of Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood. *p* 125.**

**Figure 3.4 Backgating of the CD34<sup>+</sup> Population on the FSC / SSC Dot-Plot. *p* 135.**

**Figure 3.5 Representative Experiment Demonstrating Lineage Marker Expression by Haematopoietic Progenitors. *p* 136.**



**Figure 3.6 Percentage Positive Lineage Marker Expression within the CD34<sup>+</sup> Populations of Bone Marrow, Mobilised Peripheral blood and Cord Blood.**

*p 137.*

**Figure 3.7 Mean Fluorescence Intensity of Lineage Marker Expression within the CD34<sup>+</sup> Populations of Bone Marrow, Mobilised Peripheral Blood and Cord Blood.** *p 138.*

**Figure 3.8 Single Colour Immunocytometry of Cell Adhesion Molecule Expression by KG1a.** *p 143.*

**Figure 3.9 Percentage Positive Cell Adhesion Molecule Expression by Haematopoietic Cell Lines.** *p 144.*

**Figure 3.10 Mean Fluorescence Intensity of Cell Adhesion Molecule Expression by Haematopoietic Cell Lines.** *p 145.*

**Figure 3.11 Percentage Positive Lineage and Activation Marker Expression by Haematopoietic Cell Lines.** *p 146.*

**Figure 3.12 Mean Fluorescence Intensity of Lineage and Activation Marker Expression by Haematopoietic Cell Lines.** *p 147.*

**Figure 3.13 Spectral Overlap of Third Colour Reagents.** *p 151.*

**Figure 3.14 Physical Characteristics and Fluorescence of Microbeads.** *p 153.*

**Figure 3.15 Calibration using Microbeads.** *p 154.*

**Figure 3.16 Range of Fluorescence of the CD34<sup>+</sup> Population and Dependence of the Contamination of the Study Population on the Discriminatory Marker.**

*p 157.*

**Figure 3.17 Establishment of an Acquisition Gate on the FSC / FL-2 Dot-Plot.**

*p 158.*

**Figure 3.18 Light Scatter Characteristics of CD34<sup>+</sup> Subsets.** *p 162.*

**Figure 3.19 Expression of Cell Adhesion Molecules within Haematopoietic Progenitor Subsets by Three Colour Immunocytometry.** *p 166.*

**Figure 4.1 Regression Analysis Relating Actual and Predicted Cell Numbers Over the Target Range 10<sup>3</sup> to 10<sup>6</sup> Cells.** *p 190.*

**Figure 4.2 Adhesion of KG1a to Plasma Fibronectin.** *p 202.*

**Figure 6.1. M210-B4 Murine Stromal Cell Line. *p* 250.**

**Figure 6.2 Disruption of M210-B4 Stroma. *p* 252.**

**Figure 6.3 Human Bone Marrow Fibroblast Cultures. *p* 255.**

**Figure 6.4 Human Bone Marrow Stromal Cultures. *p* 256.**

**Figure 6.5 KG1a Labelled with Dynal Beads. *p* 274.**

**Figure 6.6 Physical and Fluorescence Characteristics of KG1a Labelled with Dynal beads. *p* 275.**

**Figure 6.7 Titration of the anti-CD34 mAb 8G12-RPE on Leucapheresis Product. *p* 279.**

**Figure 6.8 Flow Cytometry of Haematopoietic Progenitors Enriched by *MACS* and FACSsort. *p* 283.**

## **CHAPTER I. INTRODUCTION.**

### **1.1 Historical Background. p 22**

### **1.2 Haematopoietic Stem and Progenitor Cells. p 23**

- .1 Theoretical considerations. p 23.*
- .2 Definition of nomenclature. p 26.*
- .3 Functional haematopoietic progenitor assays. p 26.*
- .4 Immunophenotypic haematopoietic progenitor assays. p 28.*

### **1.3 Regulation of Haematopoiesis. p 32**

- .1 Embryonic, foetal and adult haematopoiesis. p 32.*
- .2 The architecture of the bone marrow. p 33.*
- .3 The haematopoietic microenvironment. p 36.*

### **1.4 Haematopoietic Progenitor Transplantation. p 41**

- .1 Clinical bone marrow transplantation. p 41.*
- .2 Alternative sources of haematopoietic progenitors. p 43.*
- .3 Ex vivo manipulation of haematopoietic progenitor grafts. p 46.*
- .4. Haematopoietic progenitor transplantation as a problem in cell position. p 50.*

### **1.5 Cell Adhesion and Position. p 50**

- .1 Cell adhesion molecules as determinants of position. p 50.*
- .2 Molecular biology of cell adhesion molecules. p 51.*
- .3 Conceptual problems: cell identity and position. p 58.*

### **1.6 Haematopoietic Progenitor Cell Adhesion and Objectives of the Study. p 59**

## 1.1 Historical Background.

Our current concept of a stem cell model of haematopoiesis dates to the seminal observations of Paul Ehrlich [Ehrlich, 1879; Lajtha, 1980]. Using newly developed cytochemical stains he was able to distinguish several types of cells within bone marrow smears, including large basophilic cells, which he believed to be developmental precursors of granulocytes. In 1898, Pappenheim developed more sensitive staining techniques which enabled him to distinguish more closely related cells, and to infer lineage relationships [Pappenheim, 1898]. He formulated the hypothesis that rare populations of immature cells gave rise to large populations of mature cells through sequential division and differentiation. He further suggested that committed myeloid and lymphoid progenitors were themselves derived from a common multipotential progenitor.

Evidence to support this proposition did not come until the development of murine bone marrow transplantation experiments in the late 1950's [Ford, Hamerton, Barnes *et al.* 1956]. They demonstrated that transfused bone marrow cells could rescue mice whose haematopoietic and lymphoid systems had been ablated by irradiation. Using this model, Till and McCulloch (1961) sacrificed irradiated, transplanted mice two weeks after marrow infusion, and demonstrated the presence of macroscopic colonies in the spleen, each of which contained many cells of different lineages. Furthermore, cells from these splenic colonies, when pooled and injected into a second irradiated mouse, were capable of generating further splenic colonies, and rescued the mice from bone marrow failure. From these observations Till and McCulloch drew three conclusions: first, that each colony had arisen by repetitive division and differentiation from a single cell (which they termed a spleen colony forming unit (CFU-S)); second, that at least some CFU-S gave rise to mature cells of several different lineages; and third, that the CFU-S pool was self-renewing and capable of long-term support of haematopoiesis. Lajtha, Possi, Schofield *et al* [1969] used the pulsed incorporation of toxic doses of radioactive DNA nucleotides to demonstrate that less than 5% of CFU-S were proliferating at any one time.

More recently, Lemischka, Raulet and Mulligan [1986] have used retroviral

transfection of murine bone marrow cells with a genetic construct coding for aminoglycoside resistance (which is randomly integrated into the genome), to demonstrate that both myeloid and lymphoid cells may derive from the same precursor.

## **1.2 Haematopoietic Stem and Progenitor Cells.**

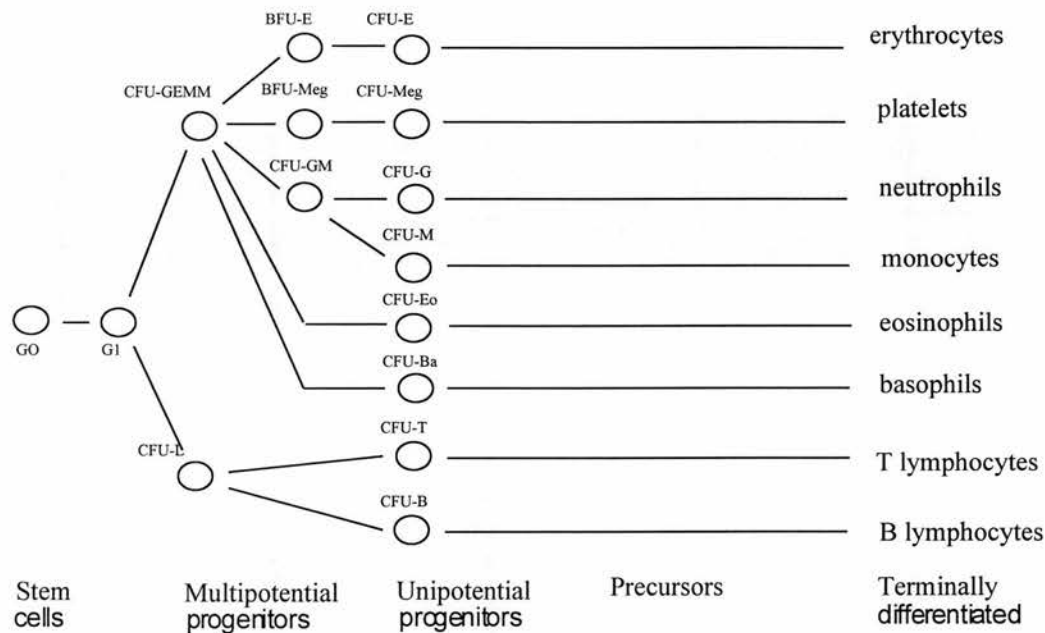
### *1.2.1 Theoretical considerations.*

The stem cell model of haematopoiesis is envisaged as a hierarchical scheme, in which cellular proliferation and differentiation proceed through an arborescent series of events, giving rise to at least 8 different cell lineages (Figure 1.1) [Spangrude, 1991]. Although the linear events of this process are recognised to form a continuum, different stages are classified into functional compartments for convenience.

Stem cells are defined as undifferentiated cells capable of self-renewal, multilineage proliferation and differentiation, long-term regeneration of tissue, and flexibility in the use of these options [Potten & Loeffler, 1990]. Haematopoietic stem cells are estimated to comprise somewhere between  $1/10^4$  and  $1/10^6$  of the nucleated haematopoietic cells in the bone marrow, and the entire adult pool may therefore comprise no more than  $1-2 \times 10^6$  cells, 90-95% of which are in quiescent phase at any one time [Lansdorp & Dragowska, 1992]. Cycling haematopoietic stem cells generate a population of committed haematopoietic progenitor cells with limited self-renewal capacity and restricted lineage potential. Together, the haematopoietic stem and progenitor cells (HPC) are termed the haematopoietic progenitor compartment, which comprises 1-2% of nucleated bone marrow cells. HPC undergo marked proliferation during the generation of precursors and terminally-differentiated cells. The turnover of haematopoietic cells in a 70kg man is estimated to be in the order of  $200 \times 10^9$  erythrocytes,  $10 \times 10^9$  neutrophils and  $400 \times 10^9$  platelets / day [Ogawa, 1993].

Many aspects of the stem cell model of haematopoiesis remain moot. Two processes may be used to explain maintenance of the stem cell pool. First, stem cells may undergo symmetric division, dividing to produce either two daughter stem cells or two more differentiated (precursor) cells. Alternatively, each stem cell division may be asymmetric, giving rise to a new stem cell and a single precursor [Holzer, Biehl, Antin

**Figure 1.1. The Stem Cell Model of Haematopoiesis.**



*Legend.* The current model of haematopoiesis is based on experiments involving *in vivo* transplantation and *in vitro* colony formation. Pluripotential haematopoietic stem cells are thought to be capable of self-renewal and multilineage proliferation and differentiation. At any one time the majority are quiescent, but proliferating cells give rise to haematopoietic progenitors of progressively more restricted lineage and proliferative potential. Morphologically identifiable precursor and terminally differentiated cells comprise up to 99% of normal marrow and peripheral blood cellularity. The marked amplification of cell numbers is a central feature of haematopoiesis.



*et al.* 1983]. Kay proposed that very few stem cells contribute to haematopoiesis at any one time, periodic activation and exhaustion of individual stem cells leading to a process of clonal succession [Kay, 1965]. Recent experimental evidence demonstrating a decrease in telomeric length with advancing gestational age suggests a finite potential for self-renewal in stem cells, and provides a rationale for a strategy of clonal succession [Hayflick, 1965; Lansdorp, Dragowska & Mayani, 1993]. Other groups have taken issue with this hypothesis and have provided evidence suggesting that a relatively large proportion of stem cells are continuously active in normal individuals [Brecher, Beal & Schneiderman, 1986; Harrison, Astle & Lerner, 1988]. McCulloch has suggested that several different differentiation pathways to a given endpoint may coexist, perhaps with predominance of more efficient or useful pathways at any one time, and variation in the blend of differentiation pathways in response to abnormal circumstance [McCulloch, 1985].

Finally, the manner in which control is exerted over haematopoietic stem cell quiescence / activation, proliferation and differentiation is unclear. Several authors have proposed deterministic models in which stem cell regulation is directed through adhesion to a specific anatomical niche (haematopoietic inductive microenvironment [Trentin, 1971 & 1989]), or through competition between humoral factors for target cells [van Zant & Goldwasser, 1979]. Ogawa has argued that the decision of a stem cell to self-renew or differentiate along a particular lineage is primarily stochastic and that the haematopoietic microenvironment fulfils a permissive role in supporting cell survival and proliferation [Nakahata, Gross & Ogawa, 1982; Ogawa, 1983 & 1993].

The manner in which the bone marrow stroma supports haematopoiesis at the molecular and cellular level is returned to in Section 1.3.3, but the distinction between a primarily instructive or a primarily selective role in control of haematopoiesis must be considered at the level of the population dynamics of cell collectives, and has important implications for interpretation of data on HPC migration and mobilisation which is returned to in Section 1.6.

### 1.2.2 Definition of nomenclature.

The nomenclature in this field can give rise to problems and to much semantic debate. I have elected to restrict the term "haematopoietic stem cell" to those conceptual entities with the functional characteristics discussed in the preceding section. The practical demonstration of "stemness" has proved elusive [Quesenberry, 1991]. Potten and Loeffler [1990] have pointed out that the defined attributes of stem cells relate to their functional potential, and in order to test whether a cell is a stem cell, it must be subjected to an environment in which it expresses that potential. In so doing the original cell is lost, and in addition, only a limited spectrum of the possible functional repertoire may be expressed.

The terms "haematopoietic progenitor compartment" and "haematopoietic progenitor cells" (HPC) are used here as a broadly inclusive reference to stem cells, multipotential progenitors and unipotential progenitors, to those cells expressing the CD34 antigen, to those cells capable of *in vitro* haematopoietic colony formation, and to those cells responsible for *in vivo* marrow repopulation in animals or man. The mapping of these theoretical, *in vitro* and *in vivo* categories is not precise, and where appropriate the limitations of a particular assay as a measure of HPC will be discussed.

### 1.2.3 Functional haematopoietic progenitor assays.

The murine spleen colony assay was the first quantitative haematopoietic progenitor assay to be developed based on the observation that intravenous injection of bone marrow cells into irradiated mice led to marrow repopulation and the development of multilineage colonies in the spleen [Till & McCulloch, 1961]. These colonies are considered to be the progeny of single clonogenic cells, and can be harvested and reinjected into a second irradiated mouse, where marrow repopulation and splenic colonies again develop. Sequential repopulation has been taken to imply a capacity for self-renewal (stem-ness), though splenic colonies have proved to be heterogeneous in their ability to sustain long term haematopoiesis [Magli, Iscove & Odartchenko, 1982]. Splenic colonies generated at day 12-14 after infusion appear to represent proliferation of more primitive colony forming cells than those generated earlier, and those cells that



generate colonies in secondary recipients may be more closely related to pluripotential haematopoietic stem cells with long term repopulating ability [Ploemacher & Brons, 1989; Jones, Wagner, Celano *et al.* 1990]. Competitive repopulation assays may be the only murine system in which we can clearly recognise and quantitate marrow repopulating cells [Harrison, Jordan, Zhong *et al.* 1993].

In 1965/66 two groups showed that it was possible to quantitate HPC by colony formation in *in vitro* semisolid culture media [Ichikawa, Pluznick & Sachs, 1966; Bradley & Metcalf, 1966]. Manipulation of the culture environment, and in particular the cytokine support, can lead to the specific expansion and enumeration of different multipotential and lineage-committed progenitors. The granulocyte-monocyte colony-forming unit assay (CFU-GM) is in most widespread use [Wu, Siminovitch, Till *et al.* 1968]. In addition, the erythroid burst-forming unit (BFU-E) [Stephenson, Axelrad, McLeod *et al.* 1971], megakaryocytic colony-forming unit (CFU-Meg) [Metcalf, MacDonald, Odartchenko *et al.* 1975], lymphoid colony-forming unit (CFU-L) [Metcalf, Warner, Nossal *et al.* 1975; Sredni, Kalechman, Michlin *et al.* 1976; Fibach, Gerassi & Sachs, 1976], and granulocyte-erythroid-monocytic-megakaryocytic colony-forming unit (CFU-GEMM) [Fauser & Messner, 1979] have been defined. Studies on highly purified populations have suggested that cells capable of long term repopulation *in vivo* are not detected by these kinds of clonogenic assay [Müller-Sieburg, Townsend, Weissman *et al.* 1988] and several assays for more primitive progenitors have been developed [Visser & Van Bekkum, 1990]. These include the human blast-colony forming assay [Nakahata & Ogawa, 1982; Rowley, Sharkis, Hattensburg *et al.* 1987; Leary & Ogawa, 1987], the human high proliferative potential colony forming assay [McNiece, Stewart, Deacon *et al.* 1989], the CFU-A assay [Holyoake, Freshney, Konwalinka *et al.* 1993], and the long term culture-initiating cell assay [Sutherland, Eaves, Eaves *et al.* 1989; Sutherland, Lansdorp, Hemkelman *et al.* 1990; Eaves, Sutherland, Udomsakdi *et al.* 1992]. The suggestion that quiescent progenitors capable of surviving *in vitro* exposure to 5 fluorouracil or 4 hydroperoxycyclophosphamide may be closely related to quiescent haematopoietic stem cells has been exploited by several groups

in the design of the pre-CFU assay [Smith, Gasparetto, Collins *et al.* 1991] and the delta assay [Rice, Boiron, Barbot *et al.* 1995]. These various assays therefore provide complementary information on HPC potentiality [Gordon, 1993a].

Xenogeneic murine bone marrow transplantation has been used as an *in vivo* model of human transplantation. Human bone marrow administered by intravenous infusion to irradiated, immunodeficient (scid or nod / scid) mice will engraft the bone marrow, and lead to multilineage haematopoietic reconstitution if other human tissues or exogenous human haematopoietic cytokines are provided [Lapidot, Pflumio, Doedens *et al.* 1992; Lapidot, Pflumio & Dick, 1993]. Several other "humanised" animal models have recently been developed which may prove useful in the exploration of human lymphohaematopoiesis [Srour, Hoffman & Zanjani, 1992].

#### *1.2.4 Immunophenotypic haematopoietic progenitor assays.*

The description of CD34 antigen expression by haematopoietic stem and progenitor cells [Katz, Tindle, Sutherland *et al.* 1985], has proved instrumental in permitting a phenotypic approach to the quantitative and qualitative study of HPC. The CD34 antigen is a heavily glycosylated type I integral membrane phosphoglycoprotein, with a strong negative charge [Civin, 1992]. Sialic acid residues contribute to an anomalous electrophoretic mobility, with an apparent molecular weight of 105-120kD [Molgaard, Brown, Simmons *et al.* 1989]. The molecular weight of the CD34 molecule predicted from the sequences of the human and murine CD34 cDNA is approximately 40kD. The function of the molecule is uncertain. The genetic sequence is unique, with no close homology to any other human molecule although there are some structural similarities to the leukosialin (CD43) family of cell surface mucins. The human gene is localised on chromosome 1q32, which is not a frequent breakpoint in haematological malignancies [Molgaard, Brown, Simmons *et al.* 1989]. The intracellular tail is a substrate for protein kinase C and other protein kinases, suggesting a role in signal transduction [Majdic, Stöckl, Pickl *et al.* 1994]. The highly sialated, negatively charged, N terminal extracellular portion of the molecule adopts an extended configuration, projecting 45nm from the cell membrane, and may prevent non-specific adhesion of cells [Civin,

1992; Greaves, Brown, Molgaard *et al.* 1992; Delia, Lampugnani, Resnati *et al.* 1993]. Alternatively, it has been suggested that the molecule acts as a scaffold to present cell-type specific carbohydrate determinants to selectins [Williams, 1991]. Recently it has been shown that vascular endothelial CD34 acts a ligand for L-Selectin, though apparently haematopoietic CD34 does not function in this way [Baumhueter, Singer, Henzel *et al.* 1993].

A large number of mAbs to the CD34 antigen have now been described [Civin, Trischmann, Fackler *et al.* 1989; Egeland, Steen, Tjonnfjord *et al.* 1993], recognising at least three discrete groups of epitopes [Lansdorp, Dougherty & Humphries, 1989]. The epitopes recognised by My10, BI-3C5, 12-8 and ICH3 are dependent on membrane-distal sialic acid residues, and are removed by *Vibrio cholerae* neuraminidase and by *Pasteurella haemolytica* O-sialoglycoprotease (Class I). QBEND 10 binds to a distinct epitope which is not neuraminidase sensitive, but remains O-sialoglycoprotease sensitive (Class II). The epitopes recognised by Tük-3, p115.2 and 8G12 are resistant to both enzymes (Class III), and are thought to be membrane-proximal [Sutherland & Keating, 1992; Sutherland, Marsh, Davidson *et al.* 1992]. Cross-linking of CD34 by QBEND10 but not by mAbs to proximal epitopes has recently been demonstrated to induce homotypic aggregation of the cell line KG1a through activation of CD18 [Majdic, Stockl, Pickl *et al.* 1994; Traore & Hirn, 1994].

The value of CD34 to the investigator is as a marker of primitive differentiation rather than of lineage specificity [Peschel & Köller, 1989; Holyoake & Alcorn, 1994]. Approximately 1.5% of nucleated bone marrow cells and 0.1% of nucleated peripheral blood cells are CD34<sup>+</sup> [Civin, Trischmann, Fackler *et al.* 1989; Bender, Unverzagt & Walker, 1991], and purified populations have a blast or early lymphoid cell morphology. Virtually all *in vitro* colony forming cells are found within this population, including unipotent and multipotent colony forming cells, blast colony forming cells and long term culture- initiating cells. *In vivo* CD34<sup>+</sup> populations have proved capable of long-term reconstitution of haematopoiesis and immunity in primates and man [Berenson, Andrews, Bensinger *et al.* 1988; Berenson, Bensinger, Hill *et al.* 1991; Shpall, Jones, Bearman *et al.* 1994]. Outwith the

lymphohaematopoietic system, human CD34 expression is restricted to microvascular endothelial cells, basement membrane structures at a variety of anatomical sites, and stromal precursors [Simmons & Torok-Storb, 1991a]. 40% of acute myeloid leukaemias are CD34<sup>+</sup>, as are 60% of B cell acute lymphoblastic leukaemias, and 1-5% of T cell acute lymphoblastic leukaemia. Chronic myeloid leukaemia chronic phase progenitor cells are also CD34<sup>+</sup>. Conversely chronic lymphatic leukaemias, lymphomas, and non-haematolymphoid malignancies are CD34<sup>-</sup>. The acute myeloblastic cell lines KG and KG1a are 70-90% CD34<sup>+</sup> [Peschel & Köller, 1989]. The murine homologue of CD34 has recently been described as showing a similar pattern of tissue expression namely HPC, vascular endothelium and the budding edge of new capillaries during angiogenesis [Baumhueter, Kyle, Dybdal *et al*, 1994], brain and embryonic fibroblasts [Brown, Greaves & Molgaard, 1991; Young, Baumhueter & Lasky, 1995].

The CD34<sup>+</sup> cell population is heterogeneous. Dense CD34 expression occurs in the earliest progenitors, with a progressive reduction with maturation, such that some of the later progenitors may be operationally defined within the CD34<sup>-</sup> population during immunocytometric analysis. The most primitive HPC fall within the top 2% of the CD34 fluorescence profile [Sutherland, Eaves, Eaves *et al*. 1989]. In addition, this population preferentially lacks expression of CD38 [Terstappen, Huang, Safford *et al*. 1991] and / or HLA-DR [Civin & Loken, 1987; Srouf, Brandt, Briddell *et al*, 1991]. Terstappen *et al* [1991] have used multiparameter flow cytometry to demonstrate that CD34<sup>hi</sup> CD38<sup>-</sup> cells comprise 1% of HPC in the bone marrow, lack differentiation markers, and are a homogeneous population of primitive blast cells by morphology with intermediate FSC and low SSC characteristics. Individual sorted cells gave rise to primitive blast colonies [Leary & Ogawa, 1987] and could sustain repetitive replating, implying a capacity for self-renewal. Expression of the CD38 antigen is an early event in differentiation, and CD34<sup>+</sup> CD38<sup>hi</sup> cells co-express early myeloid, erythroid, B or T lymphoid lineage-associated antigens. CD34<sup>+</sup> cells display low expression of CD45 common determinants [Shah, Civin & Loken, 1988]. CD45 isoforms are generated by differential mRNA splicing, though the biological functions are unknown [Thomas,



1989; Caldwell, Patterson, Toalson *et al.* 1991]. Several groups have provided evidence of mutually exclusive sub-populations of CD34<sup>+</sup> cells defined by expression of the CD45RO and CD45RA isoforms. CD45RO contain pluripotential and erythroid precursors, CD45RA myeloid and B lymphoid precursors. [Lansdorp, Sutherland & Eaves, 1990; Fritsch, Buchinger, Printz *et al.* 1993b; Craig, Poppema, Little *et al.* 1994]. Primitive HPC also lack lineage specific markers [Andrews, Singer & Bernstein, 1989 and 1990; Smith, Gasparetto, Collins *et al.* 1991], but express the Thy-1 antigen at low density [Baum, Weissman, Tsukamoto *et al.* 1992; Craig, Kay, Cutler *et al.* 1993]. The majority of clonogenic cells have been shown to be present in the CD34<sup>+</sup> Thy-1<sup>-</sup> fraction, and the majority of long-term culture initiating cells (LTC-IC) in the CD34<sup>+</sup> Thy-1<sup>+</sup> fraction. In addition to these antigenic features, primitive HPC display low retention of the supravital mitochondrial dye Rhodamine 123 [Bertoncello, Bradley, Hodgson *et al.* 1991; Udomsakdi, Eaves, Sutherland *et al.* 1991], and of the DNA-specific dye Hoechst 33342 [Neben, Redfearn, Parra *et al.* 1991]. It has been suggested that the decreased accumulation of Rhodamine 123 and Hoechst 33342 in primitive HPC is a reflection of kinetic and metabolic quiescence, with few, or largely inactive mitochondria, and a condensed chromatin structure. However, Chaudhary and Roninson [1991] have provided evidence of high P-170 glycoprotein (P-gp) expression and activity in these cells, and have proposed that active transmembrane efflux of lipophilic compounds is responsible for this phenomenon. There is some evidence to suggest that the coordinate expression of CD34 and Pgp is restricted to quiescent stem cells [Chaudhary & Roninson, 1991; List, Spier, Cline *et al.* 1991]. Huang & Terstappen [1992; 1994] have recently suggested that the CD34<sup>hi</sup> CD38<sup>-</sup> HLA-DR<sup>-</sup> population in bone marrow contains cells capable of fibroblastic colony formation as well as haematopoietic colony formation, implying that this phenotype includes an antecedent common mesenchymal stem cell.

The more common activated and lineage-committed HPC display higher Rhodamine 123 retention, and coexpress CD38, HLA-DR and CD45RA, as well as early markers of myeloid [CD33, CD13] [Andrews, Singer & Bernstein, 1989; Terstappen, Safford & Loken, 1990], erythroid (CD71) [Loken, Shah, Dattilio *et al.*

1987a], T lymphoid (TdT, CD7, CD5, CD2) [Gore, Kastan & Civin, 1991; Terstappen, Huang & Picker, 1992], B cell (TdT<sup>+</sup>, CD10<sup>+</sup>, CD19<sup>+</sup>) [Loken, Shah, Dattilio *et al.* 1987b] or fibroblast (Stro-1) [Simmons & Torok Storb, 1991b; Gronthos, Graves, Ohta *et al.* 1994] differentiation. It is possible to further dissect HPC on the basis of cytokine receptor expression such as c-kit [Papayannopoulou, Brice, Broudy *et al.* 1991; Briddell, Broudy, Bruno *et al.* 1992; Simmons, Aylett, Niutta *et al.* 1994], enzymes such as myeloperoxidase [Strobl, Takimoto, Majdic *et al.* 1993] or differential staining with lectins [Craig, Thomas & Lansdorp, 1992].

Caution should be exercised in the interpretation of expression of a particular antigen as a lineage-specific marker. For example, CD71 (transferrin receptor) is widely expressed by proliferating cells, and is therefore only partially indicative of erythroid commitment. Although several of the other antigens noted can be regarded as lineage-specific post-commitment, uncertainty exists as to the pattern of expression on earlier progenitors. Greaves has proposed that some observations of "lineage- infidelity" in leukaemic phenotypes, may be representative of a phase of "lineage-promiscuity" amongst multipotential cells, during which lineage-specific antigens may be coexpressed prior to irreversible commitment [Greaves, Chan, Furley *et al.* 1986]. Combinations of antigenic markers may prove to be a more accurate reflection of HPC subset commitment.

### **1.3 Regulation of Haematopoiesis.**

#### *1.3.1 Embryonic, foetal and adult haematopoiesis.*

Haematopoietic stem cells are derived from haemangioblasts of the splanchnic mesoderm which forms on day 7 of gestation in the region of the primitive streak and is thought to give rise to both the vascular and haematopoietic systems [Broxmeyer, 1991; Tavassoli, 1994]. Between 7 and 8 days of gestation these cells migrate along the amniotic wall and visceral endoderm to form the yolk sac blood islands. Extraembryonic blood islands support haematopoiesis from 15 days to 6 weeks of gestation. The yolk sac microenvironment is selectively permissive for a primitive form of erythropoiesis in which cell differentiation is synchronous and the end

products are nucleated red cells. These features of primitive erythropoiesis are reminiscent of the intravascular form of erythropoiesis which occurs in amphibians and birds, in which definitive adult haematopoiesis occurs in the mesodermal region containing the dorsal aorta [Tavassoli, 1991]. Medvinsky *et al* have recently demonstrated that in embryonic mice, transitory yolk sac haematopoiesis is superseded by intraembryonic haematopoiesis arising from the axial aorta-gonad- mesonephros region, which is followed by foetal liver haematopoiesis [Medvinsky, Samoylina, Muller *et al.* 1993]. Whether a similar intermediary step occurs in man is unknown, but by 6 weeks gestation a definitive form of extravascular haematopoiesis is established in the liver and spleen. Although erythropoiesis still predominates, granulopoiesis also occurs, and cells differentiate in a non-synchronous manner. The end product of erythropoiesis is an anucleate red cell.

The bone marrow develops contemporaneously with ossification of the major bones from approximately 20 weeks onwards, and becomes the major site from approximately 2 weeks post-natal. During adult life, the bone marrow supports myeloid, erythroid, monocytic and megakaryocytic development, as well as B lymphoid development, though granulopoiesis predominates. The spleen retains a permissive haematopoietic microenvironment, and in pathological conditions such as myelofibrosis may reassert this function, though, as in foetal life, erythropoiesis predominates. In the normal adult, low levels of HPC can be detected in the circulation, and HPC migration occurs from the bone marrow to the thymus (to support T lymphoid development), and to areas in the stomach, caecum, mesentery and skin (to support mast cell development) [Trentin, 1989].

### *1.3.2 The architecture of the bone marrow.*

The principal haematopoietic tissue in man is the bone marrow, and this is the site in which the majority of HPC reside.

Ultrastructural study of human bone marrow has proved difficult due to the problems of obtaining specimens without mechanical trauma, and the impediment that bone provides to fixation for electron microscopy [Lichtman, 1991]. Most data are therefore

derived from rodent studies. The arterial supply comes from two sources. Nutrient arteries enter the marrow cavity through the diaphysis, branch to form medullary and radial arteries, which penetrate the cortical endosteum and undergo arborisation to form a canalicular system. Additional arterial blood enters the canalicular system from periosteal arterioles arising from muscular arteries. Cortical capillaries re-enter the medullary cavity, draining into a highly branching network of medullary sinuses, which themselves drain into a central sinus, and via emissary veins back into the systemic circulation. It has been conjectured that the supply of arterial blood to the marrow via the bone cortex causes a drop in  $pO_2$  which augments haematopoiesis [Bradley, Hodgson & Rosendaal, 1978; Koller, Bender, Miller *et al.* 1992]. Modulation of the vascular system through neural and humoral mechanisms may influence bone marrow perfusion, oxygenation and pH.

The luminal aspect of the medullary sinuses are lined by a continuous layer of endothelial cells, which are broad and flat, with overlapping edges but without tight junctions. The endothelial cells of marrow and liver sinusoids are actively endocytic, and show areas of marked attenuation of cytoplasm, approaching the thickness of a double plasma membrane (fenestrae with diaphragm). A fine basement membrane underlies the endothelial cell layer, which consists of a lattice of collagen type IV containing various glycoproteins and proteoglycans [Yurchenko & Schnitty, 1990]. The basement membrane in marrow sinuses is less complete than that at other vascular sites. The outer (abluminal) surface of the sinus is enveloped by a discontinuous sheath of adventitial reticular cells. These cells are thought to be fibroblast derived, and display extensive cytoplasmic processes which cover approximately 65% of the abluminal surface, and extend into the marrow cavity. Adventitial reticular cells can undergo major changes in cytoplasmic volume, and several forms of stress such as endotoxaemia or increased erythropoietin have been shown to markedly reduce the area of the abluminal surface covered by reticular cell processes [Lichtman, 1991]. Together, endothelial cells, basement membrane and adventitial reticular cells form a barrier between the blood and the extravascular haematopoietic marrow, which permits regulation of cell and macromolecular traffic between the two compartments



[Lichtman, Packman & Constine, 1989; Irie & Tavassoli, 1991].

Transmigration of the blood-marrow barrier by haematopoietic cells requires passage through a gap in the adventitial reticular cell layer, penetration of the basement membrane, and formation of a migration pore in the endothelial cell cytoplasm, often close to a cell junction. Electron microscope studies reveal holes in endothelial cell cytoplasm, though it is unclear whether these are artefactual, or sites of cells in passage which have been lost during preparation. Diaphragmatic fenestrae may also act as loci of minor resistance for cell migration, or may represent areas of early repair. Blood cell release from the marrow is highly discriminatory, with a very high marrow to blood ratio of immature cells, and a low marrow to blood ratio of mature cells. This is likely to be partially due to alteration in cell adhesion molecule expression or function during cellular differentiation (*vide infra*), and partially due to increased cell motility and deformability with maturation. The blood-marrow barrier also plays an important role in regulation of cell transmigration. Marrow sinus endothelial cells show a luminal surface charge distribution and sialic acid composition which is discontinuous and non-random. Anionic charge is less dense around surface vesicles (pits), diaphragmatic fenestrae, and cells in transit. Oligosaccharide groups (such as  $\alpha$ D mannose,  $\beta$ D galactose, N-acetylgalactosamine, and N-acetylglucosamine) are also highly expressed over most of the luminal surface of the cell, but are sparse on the abluminal surface, and over the perinuclear zone and tapering ends of the endothelial cells, and around vesicles and diaphragmatic fenestrae.

The cytoplasmic processes of adventitial reticular cells provide much of the physical support for developing haematopoietic cells, and are probably the principal fibroblast-like cells in the marrow. Fibroblasts also occur in association with blood vessels, nerves and bone, though it is unclear whether free-lying fibroblasts also occur within the marrow space. Preadipocytes and adipocytes appear to be derived from adventitial reticular cells, and are perisinusoidal. Marrow adipocytes differ from adipocytes in other tissues [Tavassoli, 1989], in that they are smaller, with a high proportion of neutral fats, little or no phospholipid, and a different fatty acid profile. They are corticosteroid, but not insulin-dependent, and do not undergo involution in response to

acute starvation. Intense haematopoiesis, however, engenders involution of marrow adipocytes, with metaplasia to adventitial reticular cells. Monocyte/macrophages and T lymphocytes are haematopoietic cells in their own right, and provide a major regulatory component of the stroma [Emerson & Artin, 1989]. Nerves accompany vascular structures, myelinated fibres may affect vascular tone, whilst some authors have suggested that non-myelinated fibres end in the marrow cavity, and may affect haematopoiesis by the release of neurotransmitters.

The extracellular matrix (ECM) is secreted by stromal cells, and consists principally of collagen types I, III, IV and V (fibroblasts) [Bentley, 1982; Gay, Prince, Zuckerman *et al.* 1989], fibronectin (fibroblasts and endothelial cells), laminin (endothelial cells), and proteoglycans (in particular chondroitin sulphate and dermatan sulphate) [Keating, Wright, Kinsell *et al.* 1984; Zuckerman & Wicha, 1983; Gordon, 1988a; Zuckerman, Prince & Gay, 1989; Clark, Gallagher & Dexter, 1992]

Haematopoietic cells develop in the extravascular space, normally in close apposition to stromal cells and extracellular matrix proteins and carbohydrates, which provide a supportive haematopoietic microenvironment. Thus granulopoiesis and B lymphopoiesis occur in close association with the cytoplasmic processes of adventitial reticular cells, erythropoiesis around a central macrophage (the "erythroblastic islet"), and megakaryopoiesis adjacent to perivascular endothelial and reticular cells. Blazsek *et al.* have identified multicellular aggregates (*hematons*) in bone marrow aspirates which contain the full range of stromal and haematopoietic cells in the spatial associations described above, and which they propose should be considered as a fundamental morphogenetic unit in mammalian bone marrow [Blazsek, Liu, Anjo *et al.* 1995].

### *1.3.3 The haematopoietic microenvironment.*

Control over proliferation, differentiation and survival of haematopoietic cells *in vivo* is effected by a complex network of stromal cells, extracellular matrix proteins and carbohydrates, cell adhesion molecules, and through the secretion and presentation of regulatory cytokines [Chabannon & Torok-Storb, 1992; Gordon & Greaves, 1989;

Gordon, 1991 & 1994; Greenberger, 1986]. Although there is continued debate as to whether self-renewal and commitment decisions by multipotential progenitors are determined by the microenvironmental or are predominantly stochastic events (*vide supra* [Ogawa, Porter & Nakahata, 1983; Ogawa, 1993]), there is little doubt that continued maturation, proliferation and survival of committed progenitors is dependent on a supportive microenvironment.

It has proved difficult to examine the structural nature of the interaction between haematopoietic and stromal cells *in vivo*. Studies of *in vitro* long-term bone marrow stromal cultures (LTBMC) have shown no clear evidence of specialised membrane structures such as desmosomes, tight junctions or gap junctions, between interacting haematopoietic and stromal cells, though junctional complexes are present between stromal cells [Dexter, Coutinho, Spooner *et al.* 1990]. This suggests that intimate haematopoietic-stromal cell contact is maintained by cell adhesion molecules (CAMs) and their ligands or counter-receptors (*vide infra*). Regulatory cytokines are not passed directly to haematopoietic cells, but are presented to them in a membrane-bound form, or are secreted in a soluble form into the local microenvironment, where they may be bound by extracellular matrix proteoglycans - particularly heparan sulphate [Gordon, Riley, Watt *et al.* 1987; Roberts, Gallagher, Spooner *et al.* 1988; Dexter, Coutinho, Spooner *et al.* 1990; Gordon, 1991]. Presentation of cytokines to haematopoietic cells by stromal cells or matrix components may provide a mechanism for spatial containment of the signal. Cytokines which diffuse outside the immediate microenvironment formed by the stromal cell-ECM-target cell complex are either highly labile and rapidly degraded, or else form a background "soup" against which specific cytokine signals must be interpreted. Clearly some cytokines do act in an endocrine manner (such as erythropoietin) or can do so under iatrogenic circumstance (*e.g.* G-CSF).

A partial list of the currently described regulatory cytokines, chemokines and other factors is presented in Table 1.1 [Moore, 1991; Barrett & Gordon, 1993]. In addition, there are a number of humoral factors more properly classified as endocrine, which have an important effect on haematopoiesis (Table 1.2). The

**Table 1.1 Regulatory Cytokines.***Growth factors.*

granulocyte-monocyte colony stimulating factor (GM-CSF)

granulocyte colony stimulating factor (G-CSF)

monocyte colony stimulating factor (M-CSF, CSF-1)

erythropoietin (EPO)

thrombopoietin (TPO)

stem cell factor (SCF) (mast cell factor, c-kit ligand)

basic fibroblast growth factor (bFGF)

flk-2 / flt-3 ligand

*Interleukins.*

Interleukin (IL) -1 $\alpha$  and -1 $\beta$ , 2 to 14.

*Interferons.*

Interferon (IF)  $\alpha$ ,  $\beta$  and  $\gamma$ .

*Transforming Growth Factors.*

transforming growth factor (TGF)  $\beta$

tumour necrosis factor (TNF)  $\alpha$  and  $\beta$

*Chemokines.*

macrophage inflammatory protein (MIP) -1 and 2 $\alpha$  and  $\beta$

platelet factor 4 (PF4)

macrophage chemotactic and activating factor (MCAF)

**Table 1.2 Endocrine Factors Contributing to Haematopoietic Regulation.**

thyroid hormones

testosterone

insulin

insulin-like growth factors 1 and 2 (somatomedins).

cytokines can be loosely classified into early, intermediate and late acting on the basis of *in vitro* studies on HPC [Ogawa, 1993]. Although it is clear that some do have relatively well defined stimulatory or inhibitory effects on restricted haematopoietic lineages, most have more complex functions. IL-4, for example, demonstrates polyfunctional stimulatory and inhibitory activity on haematopoietic proliferation [Peschel, Paul, Ohara *et al.* 1987]. Other cytokines demonstrate considerable pleiotropism outwith the haematopoietic system. For example, IL-6 activity has also been demonstrated in the immune, hepatic, nephritic and other systems [Akira, Hirano, Taga *et al.* 1990]. Finally, it has become clear from work on *ex vivo* cellular manipulation that combinations of cytokines have a much more profound effect on haematopoietic proliferation than individual agents [Haylock, To, Dowse *et al.* 1994]. Considerable subtlety and some degree of redundancy therefore exist within the microenvironmental systems which effect regulatory control over haematopoiesis [Metcalf, 1993].

Although cytokines do not fall easily into a small number of families [Groopman, Molina & Scadden, 1989; Moore, 1991], their receptors do appear to do so (Table 1.3) [Barrett & Gordon, 1993; Cosman, Lyman, Idzerda *et al.* 1990]. The haematopoietin receptor superfamily are heterodimers, consisting of an  $\alpha$  chain displaying high ligand specificity but low binding affinity, which undergoes conformational change on ligand binding allowing association with a  $\beta$  chain which clamps the cytokine (forming a high affinity receptor) and mediates transmembrane signalling. The GM-CSF, IL-3 and IL-5 receptor  $\alpha$  chains associate with, and may compete for, a common  $\beta$  chain. Different types of  $\beta$  chain are responsible for different kinds of signalling. This flexibility of association provides a basis for understanding the pleiotropic (association of a given  $\alpha$  chain with several different  $\beta$  chains), and redundant (association of several different  $\alpha$  chains with a  $\beta$  chain) nature of cytokine activity. A cell may respond to several cytokines in a similar manner or to the same cytokine in a different manner, dependent (in part) on the relative affinity of  $\alpha$ - $\beta$  binding.

Binding of a cytokine to a cytokine receptor leads to internalisation and degradation



### **Table 1.3 Haematopoietic Cytokine Receptors.**

#### *Immunoglobulin gene superfamily.*

M-CSF receptor (c-fms) (CD115)

SCF receptor (c-kit) (CD117)

IL-1 receptor (CD121)

#### *Haematopoietic receptor superfamily.*

GM-CSF receptor\* (CDw116)

IL-2 receptor (CD122)

IL-3 receptor\* (CD123)

IL-4 receptor (CDw124)

IL-5 receptor\* (CD125)

IL-7 receptor (CDw127)

EPO receptor

IF receptors (CD118 / CD119)

#### *Hybrid receptors.*

G-CSF receptor

#### *Nerve growth factor receptor superfamily.*

TNF receptor (CD120)

IL-6 receptor (CD126 / CDw130)

#### *Others.*

IL-5 receptor  $\alpha$  chain

IL-8 receptor (CDw128)

TGF $\beta$  receptor

*Legend.* For a key to the abbreviations please refer to the text and Table 1.1.

\*independent  $\alpha$  chains compete for a common  $\beta$  chain.



of the complex. An equilibrium binding affinity assumes a steady state process, but in fact constant removal of bound complex with replacement by fresh cytokine receptor means that equilibrium is rarely achieved and actual binding affinity is higher than equilibrium binding affinity. One may conclude that removal of bound receptor forms a mechanism for temporal containment of the cytokine signal.

Given the abundance of factors which can be demonstrated to affect HPC survival, proliferation and differentiation *in vitro*, it has proved remarkably difficult to determine which may be of relevance *in vivo*. Results of Northern blot analyses, reverse transcription - polymerase chain reaction analyses and bioassays in long-term bone marrow cultures have demonstrated constitutive expression of M-CSF, G-CSF, IL-6, IL-3 and c-kit [Kittler, McGrath, Temeles *et al.* 1992]. A dominant negative regulatory effect appears to be provided by TGF $\beta$  [Eaves, Cashman, Kay *et al.* 1991].

#### **1.4 Haematopoietic Progenitor Transplantation.**

##### *1.4.1 Clinical bone marrow transplantation.*

In 1990 E Donnall Thomas was awarded a Nobel Prize for his pioneering work on the development of human bone marrow transplantation [Thomas, 1994]. During this procedure autochthonous haematopoiesis is ablated by high dose chemotherapy with or without radiotherapy. Intravenous infusion of previously harvested autologous or allogeneic bone marrow, leads to reconstitution of haematopoietic and lymphoid systems by exogenous HPC. Recovery of peripheral blood erythrocytes, neutrophils and platelets is normally complete within two to three weeks, though reconstitution of the immune system can take up to 6-12 months [Atkinson, 1990; Lum, 1990]. Prompt lymphohaematopoietic recovery has been found to be related to the "dose" of HPC reinfused, with a threshold effect apparent at approximately  $2 \times 10^8$  nucleated cells/kg,  $2 \times 10^5$  CFU-GM/kg,  $2 \times 10^6$  CD34<sup>+</sup> cells/kg or  $1.2 \times 10^6$  morphological blasts/kg [Bender, To, Williams *et al.* 1992].

Over the past 20 years bone marrow transplantation has come to fulfil an important role in the management of haematological and solid malignancies, bone marrow

**Table 1.4 Current Clinical Indications for Bone Marrow Transplantation.**

*Haematological and solid malignancies.*

acute myeloid and lymphoblastic leukaemia

chronic myeloid leukaemia

myelodysplastic syndromes

multiple myeloma

breast and germ cell tumours

*Bone marrow failure.*

severe aplastic anaemia

*Inborn errors of haematopoiesis, immunity and metabolism.*

haemoglobinopathies (e.g. sickle cell disease,  $\beta$  thalassaemia)

severe combined immune deficiency (e.g. adenosine deaminase deficiency)

lysosomal storage diseases (e.g. Gaucher's disease).

---

failure, and inborn errors of haematopoiesis, immunity and metabolism (Table 1.4). The procedure is, however, associated with considerable short and long-term morbidity and mortality, which arises from several sources. First, an intensive chemo-radiotherapy conditioning regimen is required to treat endogenous disease (in the case of neoplasia), to create "space" in the marrow for incoming HPC, and to suppress the immune system which would normally reject allogeneic HPC. This engenders an obligatory period of severe marrow hypoplasia during which the patient requires intensive blood component support and protective isolation. Other systems such as the gastrointestinal tract sustain collateral toxicity and transient functional impairment. Methods of avoiding high-dose chemotherapy [Wu & Keating, 1993] or reducing or eliminating the period of marrow hypoplasia would markedly improve the safety of the procedure. Second, the use of autologous transplantation permits higher doses of chemotherapy for treatment of neoplastic

diseases, but has led to concern about the extent of neoplastic contamination in the graft. Although the majority of patients probably undergo disease recurrence arising from endogenous chemotherapy-resistant disease, reinfused cells can also contribute [Brenner, Rill, Moen *et al.* 1994; Rill, Santana, Roberts *et al.* 1994]. Methods of utilising less contaminated source material, of high-grade HPC enrichment or of neoplastic cell purging, may improve disease free survival in these patients (*vide infra*). Third, allogeneic transplantation is used in the management of some malignant diseases, but also in patients with marrow failure and inborn genetic errors. The central problem is that of immune incompatibility between donor and recipient leading to failure of engraftment or to graft versus host disease. Only 30% of the Caucasian population in this country have an HLA-matched sibling donor. The exceptional polymorphism of the HLA loci engenders great difficulty in finding HLA-matched unrelated donors, and transplants are associated with particularly high procedure-related morbidity and mortality due to graft failure and graft versus host disease. The use of foetal haematopoietic tissue or more subtle immunological modulation of the graft may mitigate these problems.

#### *1.4.2 Alternative sources of haematopoietic progenitors.*

Although HPC circulate in the peripheral blood at low levels in the normal adult [Chervenick & Boggs, 1971; Kurnick & Robinson, 1971], elevated levels occur under a variety of physiological, pathological and iatrogenic circumstances (termed *mobilisation*: Table 1.5). Minor and transient elevation of HPC occurs following exercise and physiological stress [Barrett, Longhurst, Sneath *et al.* 1978]. Much more marked elevation in circulating HPC occurs during the recovery phase following marrow suppression by chemotherapy, usually just preceding recovery of peripheral blood neutrophils and platelets. The extent of elevation is dependent on the nature of the disease, the chemotherapeutic regimen, and the extent of prior exposure to chemotherapy [Craig, Smith, Parker *et al.* 1992]. As a generalisation, the more intensive the chemotherapeutic regimen, the greater the rebound. Peripheral blood leucocytes can be harvested by apheresis and cryopreserved for future use [Hénon,

**Table 1.5 Conditions Giving Rise to Elevation of Circulating Haematopoietic Progenitors.**

foetal and neonatal life.

exercise, ACTH, hydrocortisone, endotoxin

dextran sulphate

recovery phase following chemotherapy.

haematopoietic cytokines: G-CSF, GM-CSF, IL-3.

---

Butturini & Gale, 1991; Craig, Turner & Parker, 1992; Hénon, 1993; Holyoake & Franklin, 1994]. More recently it has been found that the administration of recombinant human cytokines (in particular IL-3, GM-CSF and G-CSF) *in vivo* leads to substantial elevation of circulating HPC. The use of both these modalities in conjunction has a synergistic effect on circulating HPC levels, and has led to the development of high dose chemotherapy and cytokine regimens with the express intention of mobilising HPC for leucapheretic harvest [Siena, Bregni, Brando *et al.* 1989; Craig, Anthony, Stewart *et al.* 1992]. The use of circulating HPC for transplantation has a number of potential and actual advantages over bone marrow HPC. First, the range of patients amenable to harvest may be broadened, a general anaesthetic is avoided, and patients with disease or fibrosis involving the pelvic marrow may be harvested. Second, the rate of recovery of peripheral blood neutrophil and platelet counts is more rapid, leading to reduced procedure-related morbidity and mortality. Third, it is possible that peripheral blood harvests have less neoplastic contamination than bone marrow harvests, though no clinical studies have yet shown an advantage in terms of disease-free survival.

Peripheral blood also contains elevated levels of HPC and immature cells at birth. Fifty to 150mls of blood can be salvaged from the placenta following delivery of the child and clamping of the cord. There has been substantial recent interest in harvest and storage of umbilical cord blood, and around 50 cord blood HPC transplants have been carried out, almost all to children [Broxmeyer, Douglas, Hangoc *et al.* 1989;

Broxmeyer, Kurtzberg, Gluckman *et al.* 1991; Broxmeyer, Hangoc & Cooper, 1992; Nicol, Hows & Bradley, 1994]. There are several arguments in favour of the development of umbilical cord blood banking for allogeneic HPC transplantation; expansion of allogeneic HPC availability, avoidance of general anaesthesia and bone marrow aspiration of the donor, and the relative immaturity of the neonatal immune system, which gives rise to minimal graft versus host disease - even following transplantation between MHC incompatible individuals. The major concern with regard to umbilical cord blood transplantation is that there may be insufficient HPC in the harvest to effect long-term reconstitution in an adult recipient.

Some preliminary work has been done in the field of *in utero* transplantation. The foetus is so immunologically immature that it will accept HPC introduced into the umbilical cord or peritoneal cavity without a requirement for bone marrow conditioning [Touraine, Raudrant, Royo *et al.* 1991; Zanjani, Pallavicini, Ascensao *et al.* 1992]. Although this is a potentially useful procedure in some genetic conditions, adult derived HPC harvests usually contain immunologically aggressive T lymphocytes, which lead to fatal graft versus host disease if transplanted into a foetus. Several routes are available to reduce the immunological activity of the donor harvest including *ex vivo* CD34 enrichment or T cell depletion, the use of early umbilical cord blood, or the use of homogenised early foetal liver. Grafts derived from foetal liver are rich in primitive HPC and contain immature T cells, and can be used for transplantation both *in utero* and in the neonate. Successful foetal liver and / or thymic transplants have been carried out in children with severe congenital immunodeficiencies or congenital enzyme deficiencies [Touraine, Raudrant, Royo *et al.* 1991]. There is no convincing evidence of successful engraftment in children and adults with aplastic anaemia and leukaemia, probably due to the more intense immunosuppression required [Gale, 1992].



#### 1.4.3 *Ex vivo* manipulation of haematopoietic progenitor grafts.

Until recently the extent of *ex vivo* manipulation of the HPC harvest has been limited to separation of the mononuclear cells (MNC) and cryopreservation of the product. Routine assessment of the viability and HPC content of the harvest has utilised the CFU-GM assay. Technological advances over the last few years promise a revolution in assessment and *ex vivo* manipulation of the graft. In the first instance, CD34 immunocytometry has been shown to correlate with CFU-GM, and to be an effective method of monitoring HPC in the bone marrow and in the peripheral blood following recovery from chemotherapy. This is a more global, rapid and reproducible assay for HPC enumeration than CFU-GM, and has superseded the latter in the clinical practice of some groups [Siena, Bregni, Brando *et al.* 1991a; Siena, Bregni, Belli *et al.* 1992]. It is possible that increasing sophistication in immunocytometry technique will allow more detailed monitoring of CD34 subsets, proliferation and apoptosis.

Expression of the CD34 antigen has also been used extensively as a vehicle for HPC enrichment. Several approaches to positive selection have been developed and these are discussed in more depth in Chapter 6.6 [Visser & Van Bekkum, 1990; Wunder & de Wynter, 1994]. Studies by Berenson *et al* have shown that CD34<sup>+</sup> purified cells successfully engraft bone marrow in both primates and man [Berenson, Andrews, Bensinger *et al.* 1988; Berenson, Bensinger, Hill *et al.* 1991]. The systems marketed by CellPro, Baxter (Immunex) and Applied Immune Sciences are currently licensed for clinical use. It is claimed that these systems yield a product which has 50 to 90% CD34<sup>+</sup> purity, though this level of enrichment has proved difficult to achieve in independent laboratories [Wunder & de Wynter, 1994]. Potential advantages of using enriched products are a reduction in the volume of cells stored (with reduced storage costs and recipient cryoprotectant exposure), reduction in T cell content of the graft (*vide infra*) and purging of CD34<sup>+</sup> neoplastic cells. The predominant drawbacks are the open nature of currently available enrichment systems, poor characterisation of the effect of the enrichment on HPC subset balance and long-term viability, and expense. Interest in the field of autologous transplantation for haematological and solid malignancies has focused on the issue of neoplastic cells contaminating the graft.



Impetus has been added to the field by the discovery that in some myeloid and lymphoid malignancies neoplastic contamination can be detected in autologous grafts by the polymerase chain reaction (though not necessarily by less sensitive methods such as Southern Blot) [Craig, Langlands, Parker *et al.* 1994; Cole-Sinclair, Foroni, Wright *et al.* 1993], and the demonstration by Brenner *et al.* that neoplastic contamination can give rise to patient relapse in acute leukaemia [Brenner, Rill, Moen *et al.* 1994] and solid tumours [Rill, Santana, Roberts *et al.* 1994]. Several approaches have been explored in attempts to purge the graft, including CD34 positive selection, negative selection of neoplastic cells using mAbs or lectins to tumour-specific antigens, incubation of the graft with stromal layers, incubation with chemotherapeutic agents, activation of T and NK cells with interleukin 2, and latterly attempts to induce neoplastic cell apoptosis using antisense oligonucleotides. Experimental work has shown variable degrees of success with these methods, but none has yet translated into an improvement in clinically relevant end-points.

Allogeneic transplantation is associated with a very high procedure-related morbidity and mortality, dependent on the extent of immune incompatibility between donor and recipient. Clinically, this presents as a primary failure of engraftment (host rejection of the graft), or as acute or chronic graft versus host disease. It has been shown that T cell depletion of the graft virtually eliminates graft versus host disease, but unfortunately the potential clinical benefit is offset by increased incidence of graft failure, and increased relapse of neoplastic (particularly leukaemic) disease. Two approaches bear promise for the future. First, it may be possible to selectively and quantitatively deplete T cell subsets from the allogeneic graft, with the aim of modifying the intensity of the graft versus host disease, or discriminating graft versus host from a graft versus leukaemia effect. Second, it may be possible to mimic a graft versus leukaemia effect in the autologous setting through activation of T and NK cells by immune cytokines such as interleukin 2. Current experience with IL-2 suggests that immunotherapy is more likely to be effective in the setting of minimal residual disease than in the management of bulk disease. The most promising current approach combines high dosage chemo-radiotherapy and autologous bone marrow rescue with

administration of IL-2 either to the graft *ex vivo*, or to the patient post-transplant, or both [Heslop, Duncombe, Reittie *et al.* 1991].

The availability of recombinant human cytokines has created the possibility of *ex vivo* expansion of the HPC population. Current experimental data suggests that the use of combinations of between 4 and 6 cytokines effect marked expansion in the CD34<sup>+</sup> population, and in more mature precursors and terminally differentiated cells [Moore, 1991; Haylock, To, Dowse *et al.* 1994]. The hope is that *ex vivo* HPC expansion will allow transplantation from relatively small numbers of HPC (*e.g.* from an umbilical cord harvest), or will improve the rapidity of engraftment, allowing reduction or elimination of the pancytopenic period. A theoretical concern is that the haematopoietic stem cell pool in the graft may be exhausted through cytokine-driven proliferation and differentiation, leading to long-term graft failure [Moore, 1992]. To date, expanded HPC populations have not been transplanted into patients, though this development seems imminent.

Finally, HPC form an attractive target for gene transduction, for the correction of a wide range of genetic and acquired diseases of haematopoiesis, immunity and metabolism (Table 1.6) [Miller, 1990; Larrick & Burck, 1991; Karlsson 1991]. Successful transduction and transplantation of HPC may theoretically lead to long-term expression of a construct by derivative progeny belonging to several lineages. The problems are several fold. First, current approaches to gene transduction are non-targeted, and therefore require highly enriched HPC populations to achieve satisfactory vector to target cell ratios. At present, the most widely used transduction vehicles are retroviral which have a high transfection efficiency, but are limited in the size of passenger construct and to transfection of actively replicating cells. There are associated risks of generation of replication-competent retrovirus by recombination, and of insertional mutagenesis due to random insertion of the construct into the genome. Alternative methods using other viruses (such as adenoviruses and adeno-associated virus) or physical transfection vectors (such as electroporation, liposomes or macromolecular complexes) are under scrutiny. The priorities are to achieve high efficiency transfection of rare, quiescent haematopoietic stem cells, with larger genetic

**Table 1.6 Candidate Diseases for Correction by Gene Transfer into Haematopoietic Progenitors.**

*Class I:* expression of the genetic defect restricted to lymphoid cells *e.g.* non-ADA deficient severe combined immune deficiency, X-linked agammaglobulinaemia.

*Class II:* expression restricted to haematopoietic and lymphoid cells *e.g.* Wiskott-Aldrich syndrome, Chediak-Higashi syndrome.

*Class III:* expression restricted to haematopoietic cells *e.g.*  $\alpha$  and  $\beta$  thalassaemia, sickle cell disease and other haemoglobinopathies, chronic granulomatous disease, platelet storage pool disorders, osteopetrosis.

*Class IV:* generalised expression of the genetic defect, clinical manifestations restricted to lymphohaematopoietic cells *e.g.* adenosine deaminase deficiency, purine nucleoside deficiency, Fanconi's anaemia.

*Class V:* generalised expression with generalised clinical manifestations *e.g.* Lesch-Nyhan syndrome, mucopolysaccharidoses, mucopolipidoses, lysosomal storage disorders such as Gaucher's disease and Niemann Pick disease.

*Class VI:* defective gene not normally expressed by lymphohaematopoietic cells *e.g.* haemophilia A and B, von Willebrand's disease,  $\alpha_1$  antitrypsin deficiency.

---

constructs which may need to contain regulatory sequences for appropriate expression, and genome targeting sequences for homologous recombination.

Finally, it should be clear that successful HPC gene transfection *ex vivo* may not translate into stable morphological reconstitution *in vivo*. Primary failure of the transfected HPC graft may occur if the cells have entered apoptosis. Secondary failure may occur if primitive quiescent stem cells have not been transduced, or if there is successful competition from autochthonous HPC. Indeed expression of a neoantigen by the transduced population may lead to immunological rejection of the graft. The manner in which HPC are manipulated *ex vivo* may prove equally as important as the structure of the genetic construct and the nature of the vector in achieving successful HPC gene therapy.

#### *1.4.4 Haematopoietic progenitor transplantation as a problem in cell position.*

Much of the focus of academic and clinical research over the past 20-30 years has reflected on the nature and identification of haematopoietic stem cells, and latterly on the control of proliferation and differentiation by soluble cytokines. It is clear that HPC physiology is dependent on context, provided *in vivo* predominantly by the bone marrow microenvironment. As discussed above HPC are not merely sessile but are capable of ontogenic and adult migration, selective homing to the bone marrow and mobilisation to the peripheral blood under a variety of physiological, pathological and iatrogenic circumstances. The determinants of HPC position are therefore not simply of cursory interest, but are likely to prove of central importance to a fuller understanding of HPC physiology, and are of relevance to clinical HPC transplantation and *ex vivo* manipulation.

### **1.5 Cell Adhesion and Position.**

#### *1.5.1 Cell adhesion molecules as determinants of position.*

Close cell-cell contact between mammalian cells is opposed by mutual repulsion thought to be conveyed by negatively charged sialic acid residues of molecules such as leukosialin and CD34 [Bell, Dembo & Bongrand, 1984; Greaves, Brown, Molgaard *et al.* 1992]. Cell-cell and cell-matrix adhesion are active functional processes involving membrane expression of cell adhesion molecules (CAMs). Individual CAMs demonstrate high specificity in the cognate ligands or counter-receptors to which they bind.

Three types of CAM-ligand binding have been described [Alberts, Bray, Lewis, *et al.* 1989]. Homophilic binding involves binding of a cell surface molecule to a similar molecule on an apposing cell (trans-binding), or sometimes on the adjacent membrane of the same cell (cis-binding). Members of the cadherin family (Table 1.7) involved in the assembly of adherins and desmosomal junctions in epithelial sheets, and the immunoglobulin gene superfamily members NCAM and PECAM-1 can bind in this way.

Most cell adhesion molecules, such as integrin family members, are involved in

**Table 1.7 Families of Cell Adhesion Molecules.**

cadherin superfamily.

immunoglobulin gene superfamily (Table 1.8)

integrin superfamily (Table 1.9)

selectin family (Table 1.10)

proteoglycan analogues (Table 1.11)

CD36 / LIMP II family (Table 1.11)

---

heterophilic binding, *i.e.* bind to a counter-receptor of different structure on another cell, or to an extracellular ligand such as collagen or fibronectin. Members of the selectin family and HCAM bind to carbohydrate moieties on other cells or within the extracellular matrix.

Occasionally, cell adhesion molecules bind via a multivalent link molecule, for example, the binding of platelet gpIIb/IIIa complex to dimeric fibrinogen, or the binding of CD36 to thrombospondin.

Most cell adhesion molecules are transmembrane proteins, consisting of an extracellular domain which is involved in ligand or counter-receptor binding, a hydrophobic transmembrane domain, and an intracellular domain which is often linked to the cytoskeleton. Bidirectional signal transduction can therefore occur through many of these molecules. Changes in CAM expression are achieved through alteration in local density or overall surface expression, or modulation of the binding affinity of the molecule by modification of its chemical structure and / or electrical charge *in situ*.

### *1.5.2 Molecular biology of cell adhesion molecules.*

There are at least five families of cell surface proteins involved in cell adhesion (Table 1.7) [Long, 1992]. These are reviewed in more depth in an appended paper (Appendix 5). The cadherin family includes at least three related molecules involved in calcium dependent, cell-cell homophilic binding E- cadherin (uvomorulin),





P-cadherin, and N-cadherin [Takeichi, 1988; Takeichi, 1990; Flemming, 1990; Hynes 1992a]. Cadherins are fundamental in establishing and maintaining boundaries between multicellular structures during embryogenesis and in maintaining cellular cohesion in epithelial derived tissues. There is no evidence that Cadherins are expressed by haematopoietic tissues.

Members of the immunoglobulin gene superfamily [Williams, 1987; Williams & Barclay, 1988] include adhesion molecules which bind counter-receptors or structural ligands, antigen-specific receptors such as the T cell receptor and surface immunoglobulin, major histocompatibility complex molecules, neurologic-expressed antigens, and cytokine receptors (Table 1.8). The molecules of this family are characterised by variable numbers of immunoglobulin domains, consisting of two sheets of anti-parallel  $\beta$  pleated strands, each containing 90-100 amino acids, and an interdomain disulphide bond. Most members of the immunoglobulin gene superfamily are involved in calcium independent heterophilic binding. The prototypic molecule of this family is NCAM (CD56) [Edelman, 1987 and 1988], expression of which has not, however, been observed in HPC [Saeland, Duvert, Caux *et al.* 1992; Bender, Unverzagt, Walker *et al.* 1991].

Intercellular cell adhesion molecule, ICAM-1 (CD54), has five immunoglobulin domains, and is expressed by a wide variety of cells, including HPC [Arkin, Naprstek, Guarini *et al.* 1991; Saeland, Duvert, Caux *et al.* 1992]. ICAM-1 and two closely related immunoglobulin family genes ICAM-2 (CD102) and ICAM-3 (CD50), bind to the leukocyte  $\beta_2$  integrin LFA-1. HPC expression of PECAM-1 (CD31) expression has been described by Simmons *et al* [Simmons, Niutta, Ashman *et al.* 1992; Watt, Williamson, Genevier *et al.* 1993], with homophilic affinity for PECAM-1, and heterophilic affinity for sulphated glycosaminoglycans such as heparin and heparan sulphate [De Lisser, Newman & Albelda, 1994].

HPC expression of LFA-3 (CD58) has also been described [Saeland, Duvert, Caux *et al* 1992] and is a counter-receptor to LFA-2 (CD2) expressed almost exclusively by T cells. The majority of HPC also express HLA-DR [Srour, Brandt, Briddell *et al.* 1991]. Whether these molecules are involved in antigen presentation



**Table 1.8 Members of the Immunoglobulin Gene Superfamily.**

<i>Cell adhesion molecules.</i>	<i>Nervous system.</i>
ICAM-1 (CD54), -2 (CD102)	NCAM (CD56)
and -3 (CD50)	myelin-associated glycoprotein
LFA-3 (CD58)	peripheral myelin glycoprotein
PECAM-1 (CD31)	Thy-1* (CDw90)
VCAM-1 (CD106)	OX-2*
 <i>Immune system.</i>	 <i>Cytokine receptors.</i>
TCR and Ig	platelet-derived growth factor receptor
MHC Class I and II	carcinoembryonic antigen (CD66e)
$\beta_2$ microglobulin	SCF receptor (c-kit)
CD4 and CD8	M-CSF receptor (c-fms) (CD115)
LFA-2* (CD2)	
Poly-Ig receptor	
Fc.R	

*Legend.* \* expressed by both immune and neurologic cells.

or in regulation of HPC proliferation by T lymphocytes (or both) is unclear [Pantel & Nakeff, 1993].

Two members of the immunoglobulin gene superfamily are expressed by endothelial cells: ICAM-2 (CD102), which is constitutively expressed, and VCAM-1 (CD106), which is expressed in response to inflammatory stimuli, and binds the  $\beta_1$  integrin, VLA-4 (CD49d/CD29).

The integrins comprise a large family of transmembrane sialoglycoproteins with widespread tissue distribution, and involvement in both cell-cell and cell-matrix interaction (Table 1.9) [Hynes, 1987; Hemler, 1988; Albelda & Buck, 1990; Ruoslahti, 1991; Hynes, 1992]. Molecules of this family are membrane-spanning heterodimers, with unique  $\alpha$  subunits, and a small number of  $\beta$  subunits from which

**Table 1.9 Members of the Integrin Family.**

$\beta_1$  (CD29): *VLA Family*.

$\alpha_1 \beta_1$  VLA-1 (CD49a/CD29)

$\alpha_2 \beta_1$  VLA-2 (CD49b/CD29)

$\alpha_3 \beta_1$  VLA-3 (CD49c/CD29)

$\alpha_4 \beta_1$  VLA-4 (CD49d/CD29)

$\alpha_5 \beta_1$  VLA-5 (CD49e/CD29)

$\alpha_6 \beta_1$  VLA-6 (CD49f/CD29)

$\alpha_v \beta_1$  (CD51/CD29)

$\beta_2$  (CD18) *LEUCAM Family*.

$\alpha_L \beta_2$  LFA-1 (CD11a/CD18)

$\alpha_M \beta_2$  MAC-1 (CD11b/CD18)

$\alpha_X \beta_2$  p150,95 (CD11c/CD18)

$\beta_3$  (CD61) *Cytoadhesion Family*.

$\alpha_{IIIb} \beta_3$  GPIIb/IIIa (CD41/CD61)

$\alpha_v \beta_3$  VNR (CD51/CD61)

*Legend.* The complexity of the family has been increased by the discovery of novel  $\beta$  subunits which can form alternative complexes with  $\alpha_4$ ,  $\alpha_5$  and  $\alpha_v$ .

---

subfamilies are defined. Subunits are M<sub>r</sub> 100-200,000kD in size, and considerable structural homology is evident; the various  $\alpha$  chains are 25-65% homologous, and the  $\beta$  chains 37-45% homologous. The  $\alpha$  and  $\beta$  chains of a molecule are non-covalently bound, and both contribute to ligand specificity.  $\alpha$  chains demonstrate a highly conserved binding domain to the amino-acid sequence arginine-glycine-asparagine (RGD) [Ruoslahti & Pierschbacher, 1986 & 1987] which allows many of this family to bind to extracellular matrix proteins such as collagen type I,

fibronectin, laminin and vitronectin [Hynes, 1987 and 1992; Buck & Horowitz, 1987; Humphries, 1990]. In addition, the  $\alpha$  chain contains 3-4 repeats of a divalent cation binding site ( $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ). Changes in extracellular divalent cation concentration and temperature affect the topology and functional activity of the *in situ* molecule [Graham & Brown, 1991].

The  $\beta$  chains are common to subfamilies of receptors, and are bound to the actin-based intracellular cytoskeleton by a series of link proteins which include talin, vinculin and  $\alpha$ -actinin [Alberts, Bray, Lewis *et al.* 1989]. Subfamilies are differentiated on the basis of a common  $\beta$  chain (Table 1.9).

The  $\beta_1$  subfamily [Hemler, 1990] is widely expressed, and mainly involved in binding to ECM proteins. Two members are reported to be expressed by HPC: VLA-4 (CD49d/CD29), which has dual specificity to the heparin binding domain of fibronectin and to VCAM-1 (CD106), and VLA-5 (CD49e/CD29), which recognises the cell binding domain of fibronectin [Soligo, Schiró, Luksch *et al.* 1990; Saeland, Duvert, Caux *et al.* 1992; Liesveld, Winslow, Frediani *et al.* 1993]. The VLA-4:VCAM-1 adhesion system appears to be of particular importance in the adhesion of HPC to stroma *in vitro* [Miyake, Weissman, Greenberger *et al.* 1991; Miyake, Medina, Ishihara *et al.* 1991; Miyake, Hasunuma, Yagita *et al.* 1992; Simmons, Masinovsky, Longenecker *et al.* 1992].

The  $\beta_2$  subfamily [Larson & Springer, 1990; Arnaout, 1990] are termed leucocyte cell adhesion molecules, and comprise three members with a common  $\beta_2$  chain (CD18). LFA-1 (CD11a/CD18) is expressed by all leucocytes including HPC, and is a counter-receptor for ICAM-1 and -2 as previously discussed [Soligo, Schiro, Luksch *et al.* 1990; Kansas, Muirhead & Dailey, 1990; Saeland, Duvert, Caux *et al.* 1992; Liesveld, Winslow, Frediani *et al.* 1993].

The  $\beta_3$  (cytoadhesin) subfamily comprises gpIIb/IIIa (CD41a/CD61), an important platelet adhesion molecule for fibrinogen, fibronectin, and von Willebrand Factor (vWF); and the vitronectin receptor (VNR) (CD51/CD61).

Under normal physiological conditions, integrins largely exist in an inactive form. A variety of physiological stimuli such as cytokines or cell adhesion, or

**Table 1.10 Members of the Selectin Family.**

L-Selectin (LECAM-1, MEL-14, LAM-1) (CD62L)

E-Selectin (LECAM-2, ELAM-1) (CD62E)

P-Selectin (LECAM-3, GMP-140, PADGEM) (CD62P)

---

chemical substances such as phorbol myristate acetate (PMA) or calcium ionophore, can lead to rapid activation of integrin binding through conformational changes mediated through the cytoskeleton [Makgoba & Bernard, 1993].

The selectin family are C-type lectins *i.e.* carbohydrate-binding proteins (Table 1.10) [Rosen, 1990; Watson, Kingsmore, Johnston, *et al.* 1990; Springer & Lasky, 1991; Lasky, 1991 & 1992; Bevilacqua & Nelson, 1993]. The N terminal domain is homologous with a variety of animal lectins, including hepatic galactose receptors, soluble mannose binding lectins, invertebrate lectins, and also the low affinity receptor for IgE (CD23). They are temperature insensitive, and  $\text{Ca}^{2+}$  dependent, though  $\text{Mg}^{2+}$  independent. There are three members of the family. L-selectin (CD62L) (LECAM-1, MEL-14, LAM-1) plays a central role in the regulation of lymphocyte traffic, and has been shown to be expressed by HPC by Simmons, Niutta, Ashman *et al* [1992]. E-selectin (CD62E) (LECAM-2, ELAM-1) is expressed by endothelial cells in response to inflammatory stimuli. P-selectin (CD62P) (LECAM-3/GMP-140/PADGEM), is stored in the  $\alpha$  granules of megakaryocytes and platelets, and in Weibel-Palade bodies in endothelial cells. Current evidence suggests that selectins mediate leukocyte rolling on inflamed endothelium, by recognition and presentation of oligosaccharide ligands such as Lewis<sup>x</sup> (CD15), sialyl Lewis<sup>x</sup> (CD15s), and VIM2 (CDw65) [Lawrence & Springer, 1991; Pickler, Warnock, Burns *et al.* 1991; Foxwall, Watson, Dowbenko *et al.* 1992; von Andrian, Chambers, Berg *et al.* 1993; Kuijpers, 1993] and CD34 [Baumhueter, Singer, Henzel *et al.* 1993].

The homing-associated cell adhesion molecule HCAM (CD44) (Table 1.11) [Haynes, Telen, Hale *et al.* 1989; Lewisohn, Nagler, Ginzton *et al.* 1989] represents

**Table 1.11 Other Cell Adhesion Molecules.**

*Proteoglycan analogues.*

HCAM (CD44) Family.

*CD36/LIMP-II Family.*

GP11b or IV (CD36).

---

a family of cell surface glycoproteins ranging from 80 to 200kD, most of which have hyaluronan binding affinity (Haynes, Liao & Patton, 1991; Underhill, 1992). Tissue expression is widespread on both leucocytes and epithelial cells: the molecule has been identified as of key importance in the recirculation of lymphocytes between blood and lymphoid organs, from which it derives its name. The distal portion of the CD44 extracellular domain has significant homology with cartilage-link and proteoglycan core proteins. Cartilage-link proteins stabilise large aggregates of proteoglycan core proteins by binding proteoglycan monomers to hyaluronic acid or collagen. CD44 is the principal cell surface receptor for hyaluronic acid [Miyake, Underhill, Lesley *et al.* 1990], and binds additional extracellular matrix components including fibronectin, collagen types I and VI, and a vascular endothelial molecule. At least 12 different isoforms are generated by alternative splicing leading to the addition of variable numbers of exons to the membrane proximal extracellular domain. The haematopoietic isoform (CD44H) has no additional exons, whilst the epithelial isoform (CD44E) has additional isoforms and a reduced affinity for hyaluronic acid [Dougherty, Cooper, Memory *et al.* 1994]. The products of CD44 gene activity appear to exercise many diverse functions in the cell, and at the cell surface, and disturbances in gene activity arising from derangement in the control of alternative splicing occur early in the natural history of many malignancies [Dougherty, Lansdorp, Cooper *et al.* 1991; Stamenkovic, Aruffo, Amiot *et al.* 1991; Screaton, Bell, Jackson *et al.* 1992; Matsumura & Tarin, 1993]. Monoclonal antibodies to CD44 have been shown to

blockade lymphohaematopoiesis in bone marrow stromal cultures [Miyake, Medina, Hayashi *et al.* 1990].

CD36 (GPIIb or IV) (Table 1.11) is an 88kD transmembrane glycoprotein, with substantial homology to a rat lysosomal integral membrane protein (LIMP II). CD36 acts as a cellular receptor for oxidised low density lipoprotein, for collagen and for thrombospondin [Asch, Barnwell, Silverstein *et al.* 1987; Asch, Tepler, Silbiger *et al.* 1991; Greenwalt, Lipsky, Ockenhouse *et al.* 1992]. In addition, there is evidence that CD36 is expressed by erythrocytes infected with the human malarial parasite *Plasmodium falciparum*. CD36 displays a unique structure in which both amino and carboxyl termini are intracellular, and the central region of the molecule is extracellular. The intracellular domains contain protein tyrosine kinase activity and are thought to be involved in signal transduction [Greenwalt, Lipsky, Ockenhouse *et al.* 1992].

#### *1.5.3 Conceptual problems: cell identity and position.*

It will be understood from the foregoing discussion that there is categorical overlap between cytokines and their receptors, and cell adhesion molecules and their ligands. For example M-CSF receptor (c-fms, CD115) and SCF receptor (c-kit, CD117) are classified as cytokine receptors, but are structural members of the immunoglobulin gene superfamily of cell adhesion molecules. Presentation of cytokines on the cell membrane or bound to extracellular matrix proteoglycans may principally provide a mechanism for spatial containment or modulation of the signal [Ruoslahti & Yamaguchi, 1991], but could also be conceived as a functional cell adhesion system [Nathan & Sporn, 1991]. Conversely, members of the integrin family of cell adhesion molecules are bidirectional signal transducers, and binding to a ligand may effect changes in cellular phenotype, morphology, functional activity and survival [Ruoslahti & Reed, 1994].

Moreover, both cytokines and cell adhesion molecules show evidence of pleiotropism (*i.e.* ligands expressed by multiple targets) and polyfunctionality (*i.e.* multiple functional changes effected by ligand binding). Indeed, the targets



themselves may express multiple ligands for different cytokines, cell adhesion molecules, or ECM components. Cell adhesion behaviour is likely to be determined by the composite action of a mosaic of cell adhesion molecules, expressed at variable density, distribution and activation status on the cell membrane.

Contemplating such complexity from a homeostatic view point [Bernard, 1865] gives the appearance of marked redundancy within the system. It may be more productive to view such a system as a highly interconnected network of exceptional depth and subtlety [Metcalf, 1993]. Whichever paradigm is preferred, a reductionist approach to analysing the determinants of cell adhesion may run in to difficulties with this general property of functional overlap.

Finally, the dependence of adhesion on cell phenotype, of microenvironmental signalling on adhesion and of differential gene expression on signalling, suggests a very high degree on interdependence of cell identity, function and position [Wolpert, 1969; Edelman, 1988; Dainiak, 1991; Greenwald & Rubin, 1992].

### **1.6 Haematopoietic Progenitor Cell Adhesion: The Objectives of the Study.**

HPC interact with bone marrow in three generic ways: (1) circulating HPC recognise and transmigrate the sinusoidal endothelium (homing); (2) HPC adhere to marrow stromal cells or ECM components (engraftment); and (3) HPC communicate with regulatory cells (such as T lymphocytes and monocytes) through a variety of methods including adhesion (regulation).

Although it is recognised that these processes must be mediated in principle through HPC cell adhesion molecule recognition of ligands or counter-receptors expressed by bone marrow sinusoidal endothelium, stromal cells and extracellular matrix components, the cell adhesion molecules involved and their functional roles are poorly defined. Expression of numerous CAM belonging to several molecular families has been described by various groups. These include immunoglobulin gene superfamily members ICAM-1 (CD54) [Arkin, Naprstek, Guarini *et al*, 1991; Saeland, Duvert, Caux *et al*, 1992], PECAM-1 (CD31) [Simmons, Niutta, Ashman *et al*, 1992; Watt, Williamson, Genevier *et al*, 1993; Lund-Johansen & Terstappen, 1993] and LFA-3

(CD58) [Saeland, Duvert, Caux *et al.*, 1992], the  $\beta_1$  integrins VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29) [Soligo, Schiró, Luksch *et al.*, 1990; Roseblatt, Vuillet-Gaugler, Leroy *et al.*, 1991; Saeland, Duvert, Caux *et al.*, 1992; Teixido, Hemler, Greenberger *et al.*, 1992; Liesveld, Winslow, Frediani *et al.*, 1993; Lund-Johansen & Terstappen, 1993], the  $\beta_2$  integrin LFA-1 (CD11a/CD18) [Soligo, Schiró, Luksch *et al.*, 1990; Teixido, Hemler, Greenberger *et al.*, 1992; Saeland, Duvert, Caux *et al.*, 1992; Liesveld, Winslow, Frediani *et al.*, 1993; Kansas, Muirhead & Dailey, 1990; Papayannopoulou & Brice, 1992], L-Selectin (CD62L) [Simmons, Niutta, Ashman *et al.*, 1992; Lund-Johansen & Terstappen, 1993], the HCAM (CD44) family [Lewinsohn, Nagler, Ginzton *et al.*, 1990; Kansas, Muirhead & Dailey, 1990; Saeland, Duvert, Caux *et al.*, 1992; Lund-Johansen & Terstappen, 1993] and CD36 [Long & Dixit, 1990; Greenwalt, Lipsky, Ockenhouse *et al.*, 1992] (Table 3.1). In addition to these, CD34 may itself act as a ligand for L-Selectin [Baumhueter, Singer, Henzel *et al.*, 1993], CD43 may bind ICAM-1 (CD54) [Moore, Huang, Terstappen *et al.*, 1994] and Sialyl Lewis<sup>x</sup> is known to be a ligand for P- and E-selectins [Lund-Johansen & Terstappen, 1993; Karakantza, Gibson, Cavenagh *et al.*, 1994]. CD45 and Thy-1 are known to mediate adhesion to heparan sulphate [Parish, Hogarth & McKenzie, 1988], HLA-DR may mediate adhesion to CD4<sup>+</sup> T lymphocytes and CD4 may mediate adhesion to HLA Class II receptors on other regulatory cells such as monocytes [Zauli, Furlini, Vitale *et al.*, 1994; Louache, Debili, Marandin *et al.*, 1994]. Finally, cell membrane-associated proteoglycans may mediate adhesion to fibronectin [Minguell, Hardy & Tavassoli, 1992; Verfaillie, Benis, Iida *et al.*, 1994]. Other potential cell adhesion molecules remain poorly defined [Aizawa & Tavassoli, 1987 & 1988; Tavassoli & Hardy, 1990; Hardy, Matsuoka & Tavassoli, 1991; Tavassoli & Minguell, 1991], or are of uncertain adhesive import *e.g.* the interaction of cytokine receptors such as c-kit with their membrane- or proteoglycan-bound ligands [Reisbach, Bartke, Kempkes *et al.*, 1993].

The system displays a further level of complexity. Most CAMs display both pleiotropism and polyfunctionality due to the presence of multiple structural domains within CAMs and their ligands, and diverse secondary messenger systems.

There exist, therefore, multiple potential HPC adhesive pathways, any or all of which may participate in cell homing, engraftment and regulation. This diversity may reflect heterogeneity within the CD34<sup>+</sup> population, the variety of cellular and extracellular matrix elements with which HPC engage, or the manner in which HPC-stromal adhesion is initiated and stabilised. Finally, the possibility that real functional redundancy exists within the system must be considered.

*The first objective of this study was to establish an optimal method with which to define the human HPC population and to characterise the pattern of cell adhesion molecule expression within that population.*

The mechanisms underlying HPC release from the marrow during mobilisation are unknown, and may or may not resemble the physiological release of HPC which must occur during migration. Three hypotheses were considered: first that alteration or blockade of HPC cell adhesion molecule expression occurs, either as a direct affect of the mobilising agent, or as an indirect response to humoral feedback from other cells or tissues. Second that damage to the sinusoidal endothelium occurs, leading to loss of control over cell traffic across the blood / marrow interface. Third that perturbation of HPC population dynamics as a result of increased cell proliferation or survival leads to "spill-over" of excess HPC from the marrow into the peripheral blood (Appendix 5). These possibilities are not necessarily mutually exclusive, and different mechanisms may predominate under different circumstances (Table 1.5).

If the first proposition is correct one might expect differences in CAM expression between sessile and circulating HPC, whereas if the second or third are correct one might not.

*The second objective was to determine whether differences in CAM expression could be detected between sessile and circulating HPC and if so, whether such differences could be responsible for HPC mobilisation or simply reflect more general differences in the heterogeneity of the study population.*

Phenotypic information provides, at best, circumstantial evidence for the involvement of specific cell adhesion pathways in HPC homing, engraftment and mobilisation. Semisolid clonogenic assays have been used by several groups to investigate HPC adhesion. Selective localisation of primitive HPC in stromal cultures is recognised [Coulombel, Eaves, Eaves *et al*, 1983; Gordon, Clarke, Atkinson *et al*, 1990; Liesveld, Abboud, Duerst *et al*, 1989]. Differential binding of erythroid and myeloid progenitors to collagen type I [Koenigsmann, Griffin, DiCarlo *et al*, 1992], to fibroblasts and fibronectin [Tsai, Patel, Beaumont *et al*, 1987; Coulombel, Vuillet-Gaugler, Leroy *et al*, 1988 & 1991; Verfaillie, McCarthy & McGlave, 1990] and to hemonectin [Campbell, Long & Wicha, 1990], have been described. One of the key issues is the extent to which a causal link can be drawn between the expression of a given CAM and cellular adhesion behaviour.

*The third objective was the development of an assay system which would allow the correlation of cellular CAM phenotype with functional adhesion to a variety of substrates, and which would permit analysis of the adhesion pathways involved through selective activation and blockade.*

## **CHAPTER 2. QUANTITATIVE DETERMINATION OF HAEMATOPOIETIC PROGENITORS.**

<b>2.1 Introduction.</b>	<b>p 64</b>
<b>2.2 General Methodology.</b>	<b>p 65</b>
<i>.1 Sources of human material. p 65.</i>	
<i>.2 Culture and storage of cell lines. p 66.</i>	
<i>.3 Enumeration of nucleated cells and assessment of viability. p 68.</i>	
<b>2.3 Standard Methodology for the CFU-GM Assay.</b>	<b>p 68</b>
<b>2.4 Optimisation of CD34 Immunocytometry.</b>	<b>p 69</b>
<i>.1 Background. p 69.</i>	
<i>.2 Titration and handling of monoclonal antibodies. p 76.</i>	
<i>.3 Optimisation of immunofluorescence labelling. p 80.</i>	
<i>.4 Effects of erythrocyte lysis and cell fixation on the physical and fluorescence characteristics of the study population. p 82.</i>	
<i>.5 Set-up of the flow cytometer. p 84.</i>	
<i>.6 Comparative study of available anti-CD34 monoclonal antibodies and direct / indirect systems of labelling. p 88.</i>	
<b>2.5 Determination of Resolution Limits of Immunocytometry.</b>	<b>p 94</b>
<b>2.6 Evaluation of Different Approaches to Data Analysis.</b>	<b>p 96</b>
<b>2.7 Study of the Overall Accuracy of CD34 Quantitation.</b>	<b>p 101</b>
<b>2.8 Establishment of a Correlation between CD34 and CFU-GM Assays.</b>	<b>p 103</b>
<b>2.9 Discussion.</b>	<b>p 105</b>
<i>.1 Standardisation and reproducibility of the CFU-GM assay. p 105.</i>	
<i>.2 Standardisation and reproducibility of CD34<sup>+</sup> quantitation. p 107.</i>	
<i>.3 Theoretical considerations in the definition of the CD34<sup>+</sup> population. p 109.</i>	



## 2.1 Introduction.

The starting point for study of haematopoietic progenitor cells (HPC) was the development of accurate and reproducible assays. For many years the granulocyte / monocyte colony-forming unit assay (CFU-GM) [Pluznick & Sachs, 1965; Bradley & Metcalf, 1966] has been regarded as the gold standard for clinical HPC quantitation of bone marrow and peripheral blood harvests, and for many research purposes. This is a functional assay in which the study population is incubated for 7-14 days in culture medium, serum and a cytokine source, in a support matrix of agar or methylcellulose. HPC proliferate to form colonies which are held together by the support matrix, and can be enumerated by light microscopy against a background of individual cells or precursors with very restricted proliferative capacity. In man, CFU-GM have been shown to correlate well with neutrophil recovery after bone marrow transplantation, but to a lesser degree with platelet recovery.

Clonogenic assays suffer from several limitations for routine clinical and research use, namely a high degree of dependence on the culture conditions, difficulty in achieving standardisation between laboratories, a 14 day delay in awaiting a read-out and representation of only a subset of the HPC population (namely committed myeloid progenitors). Their central strength is that they provide direct functional evidence of the presence of haematopoietic progenitors. A standard form of the CFU-GM assay was used to validate an immunocytometry assay as described below. The culture methodology is discussed in Section 2.3.

The search for a more global, rapid and standardised HPC assay has directed attention to immunocytometry. The fluorescence activated cell scanner (FACS) combines multiparameter measurements of physical properties and fluorescence on large numbers of cells, allowing greater accuracy in the enumeration and phenotypic characterisation of cell populations [Parks, Lanier & Herzenberg, 1986]. As discussed above (Section 1.2.4), the majority of human HPC express the CD34 antigen, which may justifiably be used as a broad indicator of this compartment. Here again important problems exist, this time due to the small size of the study population and low expression of the CD34 antigen, and methodological problems



such as the choice of monoclonal antibody and fluorochrome. Much care was taken to optimise and standardise CD34 immunocytometry methodology which was critical to the accuracy of the further studies, and this is detailed in Sections 2.4 to 2.6. The overall accuracy of CD34 quantitation was studied in Section 2.7 and a linear relationship between CD34<sup>+</sup> cell numbers and CFU-GM colony formation established in Section 2.8. Finally, the methodological limitations of HPC assays, and attempts to achieve reproducibility and standardisation between different European laboratories, are discussed in Section 2.9.

## **2.2 General Methodology.**

### *2.2.1 Sources of human material*

Sources of human material included (1) peripheral blood samples from normal volunteers (PB); (2) peripheral blood (PB<sup>m</sup>) and leucapheresis product (LP) from patients with Hodgkin's disease, non- Hodgkin's lymphoma, or stage III / IV breast carcinoma, undergoing peripheral blood leucocyte harvest during the recovery phase following high dose cyclophosphamide (4g/m<sup>2</sup> iv) and recombinant human granulocyte colony stimulating factor (rhuG-CSF) (5µg/kg/day sc); (3) normal bone marrow (BM) initially obtained from the sternum of patients undergoing cardiothoracic surgery or from pelvic bone fragments from patients undergoing prosthetic hip replacement. These sources proved entirely unsatisfactory however, and for the work described bone marrow aspirates had to be obtained from the posterior iliac crest of normal volunteers under local anaesthetic, or of patients undergoing general anaesthetic for non-malignant surgical procedures; (4) Placental umbilical cord blood samples after Caesarean section (CB).

Bone marrow and blood samples were judged "normal" on the strength of a normal peripheral blood count and the absence of relevant disease in the donor. 10ml samples were collected in a syringe pre-flushed with heparin, and were held in a sterile universal container containing 10ml Hanks' balanced salts solution (HBSS) with 10mM ethylenediaminetetra-acetic acid (EDTA), so that original cell counts could be calculated from actual counts made on the diluted samples. Latterly, the

anticoagulation was changed to 500iu (4mg) lithium heparin in a 20ml universal container, which was found to be more satisfactory for BM and CB samples.

The use of human material for this study was approved by Lothian Health Board, Ethics of Medical Research Sub-Committee for Medicine and Clinical Oncology under submission numbers MCO/58/91, MCO/98/91, and MCO/47/92. All samples were taken and used with the informed consent of the patient or volunteer. Personal medical information was treated as confidential, and complied with the guidelines set out by the Data Protection Act 1984.

### *2.2.2 Culture and storage of cell lines.*

The cell lines used during these studies are listed in Table 2.1. Imported cell lines were expanded in culture to establish a master cell bank, which was stored in aliquots at -160°C in liquid Nitrogen. Aliquots were thawed for use as required to maintain a working cell bank.

Suspension cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM), 10% foetal calf serum (FCS) with 1% antibiotic stock (*standard tissue culture medium*. Appendix 2) at a concentration of  $1 \times 10^6$  cells/ml. FCS was heat inactivated by incubation at 56°C for 30min. The medium was changed and cell concentration adjusted, once or twice a week as necessary. Cell manipulation was carried out using sterile technique in a laminar flow cabinet. All tissue culture media were filtered prior to use, stored at +4°C in sterile glassware, and discarded after one week. In view of recurrent problems with fungal contamination of the cultures, 50 and 250ml canted-neck tissue culture flasks were used with a vented cap incorporating a hydrophobic membrane filter, allowing continuous gaseous exchange with the incubator environment whilst maintaining a hermetic seal. The incubator was maintained at 37°C, 5% CO<sub>2</sub> and 100% humidity.

Adherent cell lines were also maintained in standard tissue culture medium and the same incubator environment, but were passaged once a week by discarding the medium, washing once in anticoagulant medium, and incubating with sufficient trypsin / EDTA to cover the surface of the plate (1-2ml in a 50ml 25cm<sup>2</sup> flask, 5ml

### **Table 2.1 Human Haematopoietic Cell Lines Studied.**

KG1a acute myelogenous leukaemia cell line [Koeffler, Biling, Lysis *et al.* 1980].

K562. chronic myelogenous leukaemia. [Lozzio & Lozzio, 1975].

HL60 acute promyelocytic leukaemia.[Collins, Gallo & Gallagher, 1977].

CEM T lymphoblastic leukaemia (Sangster, Minowada, Suciu-Foca *et al.* 1986).

NALM-6 B cell lymphoma line.

---

in a 250ml 75cm<sup>2</sup> flask). Some adherent lines such as M210-B4 detach from the surface quite easily, but remain aggregated as sheets in suspension which required further trypsin exposure. Others, such as 5637 required prolonged trypsin incubation at 37°C along with considerable shaking to detach them from the plastic surface, but achieved a single cell suspension. Satisfactory dispersion was assessed using an inverted microscope. The action of the trypsin was arrested by adding 5ml FCS, followed by centrifugation and resuspension in fresh culture medium.

Surplus cells were cryopreserved in FCS, 10% dimethylsulfoxide (DMSO) using a programmed cell freezer, and stored at -160°C in liquid nitrogen. For recovery, cells were defrosted rapidly in a 37°C waterbath, and mixed with 1ml FCS containing 100µl magnesium sulphate, 100µl DNase, and 100µl preservative-free heparin. They were allowed to equilibrate at ambient temperature for 5 min, subjected to centrifugation, and re-suspended in culture medium for 30 min. The cells were resuspended in fresh culture medium before transfer to tissue culture flasks.

Unless otherwise stated, all centrifugation was carried out at 200g for 5 min, and the supernatant removed by tube inversion or by aspiration.

### *2.2.3 Enumeration of nucleated cells and assessment of viability.*

Enumeration of nucleated cells was carried out using a Coulter ZF cell counter, following lysis of red cells by lyzerglobin, or latterly by direct analysis using a Coulter T-890. Cell concentration could be related to differential cell counts in the original specimen because a standard amount of diluent (10ml of anticoagulant medium) was used.

$$\text{Original cell concentration} = \frac{\text{measured cell concentration} \times \text{final volume}}{\text{original volume}}$$

The later use of lithium heparin as an anticoagulant obviated the need for this calculation.

Enumeration of cell lines was carried out using a Neubauer haemocytometer. This proved more useful than the automated counters in that it allowed exclusion of cell debris and non-viable cells, and an assessment of homotypic aggregation. To maintain maximal reproducibility all cell counting was carried out by the same observer.

Cell viability was assessed by trypan blue dye exclusion, a measure of membrane integrity. 50µl of cells were incubated with an equal volume of trypan blue for 5 minutes (final concentration 0.2%), and examined in a Neubauer haemocytometer using an inverted microscope. Clear cells were considered viable, dark blue cells were considered non-viable. Of importance is the observation that trypan blue exclusion will not detect the early stages of apoptosis, and is therefore a relatively crude measure of cell survival [Koury, 1992].

### **2.3 Standard Methodology for the CFU-GM Assay.**

A standard method was adopted for the CFU-GM assay. A mononuclear cell suspension was prepared over Ficoll-Hypaque (density 1.077g/dl). 10ml of the sample in anticoagulant medium (1:2 dilution) was layered carefully over 4ml of Ficoll-Hypaque, and centrifuged at 500g for 15min. The mononuclear cells were recovered from the plasma / Ficoll interface using a disposable Pasteur pipette, and the cell concentration adjusted to  $2 \times 10^6$  cells/ml in standard tissue culture medium.



200µl of medium containing  $4 \times 10^5$  cells was added to 800µl methylcellulose, 600µl FCS, 200µl of 5637 conditioned medium, 170µl IMDM, and 30µl of antibiotic stock (total 2ml) (*CFU-GM assay medium* Appendix 2).

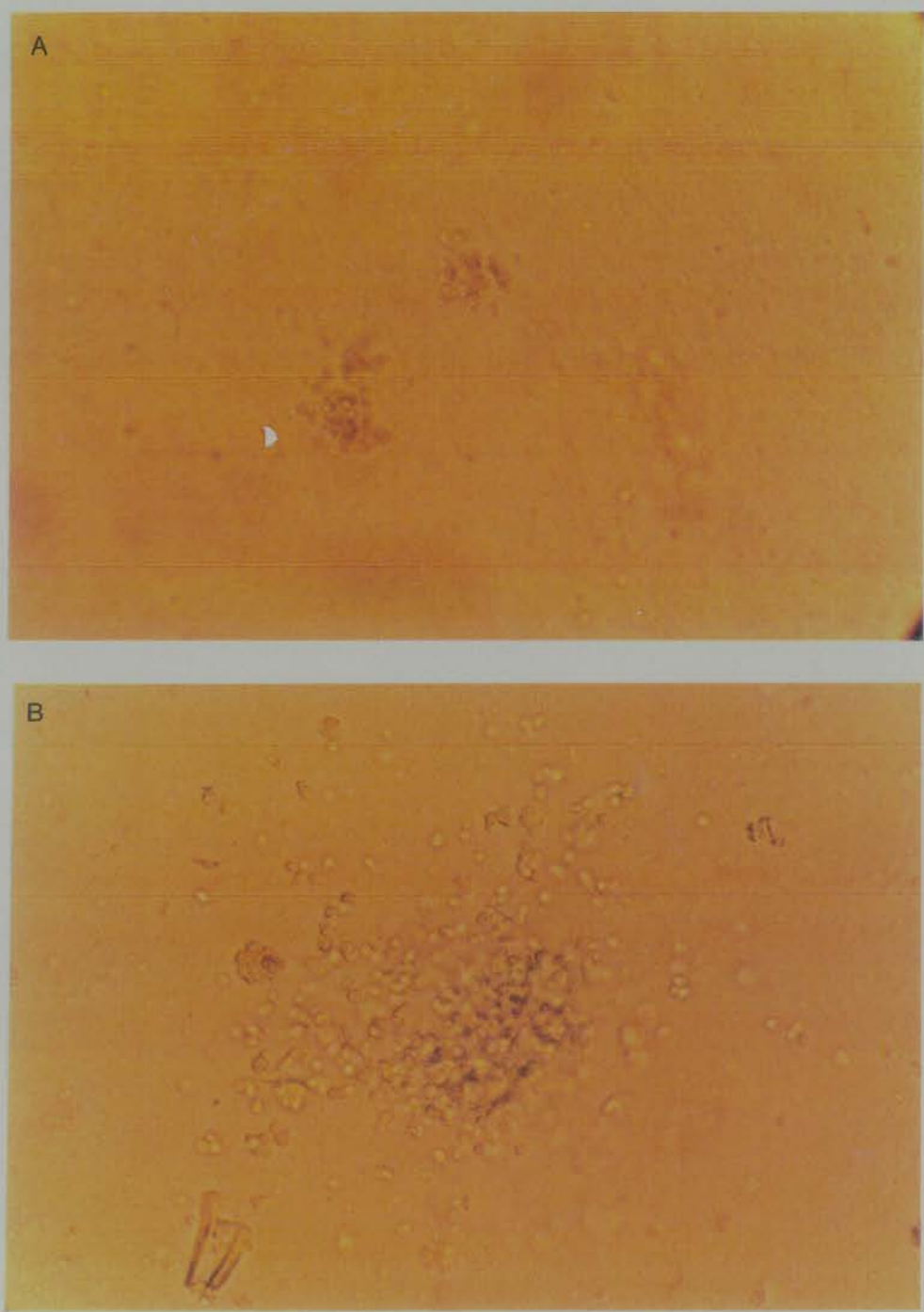
Methylcellulose was made up in the following manner: 18g of methylcellulose was autoclaved, and 450ml distilled water was filtered into a sterile bottle. The latter was heated to boiling point, the methylcellulose added, and the solution allowed to simmer for a further 10min. The temperature was allowed to drop to 37°C with magnetic stirring, and 50ml of 10 x IMDM was added. The solution was stirred for a further 15-30min at +4°C until it thickened, and was left overnight to settle [Freshney, Pragnell & Freshney, 1994]. New batches of FCS were bought in on a 6-12 monthly basis and compared for overall CFU-GM formation against each other and against previous batches. It was stored at -20°C prior to use. Conditioned medium was prepared by culture of the 5637 human bladder carcinoma-derived cell line in standard tissue culture medium to produce an adherent layer. The media was removed at weekly intervals, clarified of debris by centrifugation, and stored frozen at -70°C until required. 0.5ml of CFU-GM culture medium containing  $1 \times 10^5$  cells was added to each of 3 wells of a 24 well tissue culture cluster dish, each with an approximate surface area of 2cm<sup>2</sup>. Cultures were incubated at 37°C, 5%CO<sub>2</sub>, 100% humidity for 14 days. Clusters of 10-50 cells, and colonies of greater than 50 cells were enumerated using an inverted microscope, and the mean of the triplicate assays quoted as the result. CFU-GM clusters and colonies are illustrated in Figure 2.1.

## **2.4 Optimisation of CD34 Immunocytometry.**

### *2.4.1 Background.*

When highly purified antigens began to be used for immunisation in the 1930s and 40s it was recognised that the antibody response to a single antigen was heterogeneous *i.e.* involved the formation of complex populations of antibodies to various epitopes. Recognition of the existence of homogeneous antibody populations arose out of studies of the occurrence of Bence Jones proteinuria and serum monoclonal immunoglobulins in patients with myeloma. Kohler and Milstein

**Figure 2.1 CFU-GM Colony Assays.**



*Legend.* Cluster (A) and colony (B) formation in a day +14 CFU-GM assay (magnification x10).



[1975] were instrumental in the development of the hybridoma technique for producing monoclonal antibodies (mAbs), whereby antibody-forming spleen or lymph node cells from a mouse immunised with known antigens are fused with murine plasmacytoma cells, and the resultant clones screened for the production of antibodies of useful specificity. MAbs have provided a highly specific tool for the analysis of antigen structure and expression, and latterly a series of international workshops has established a system whereby mAbs from different sources are classified according to their antigenic specificity (cluster designation CD) [Golding, 1983].

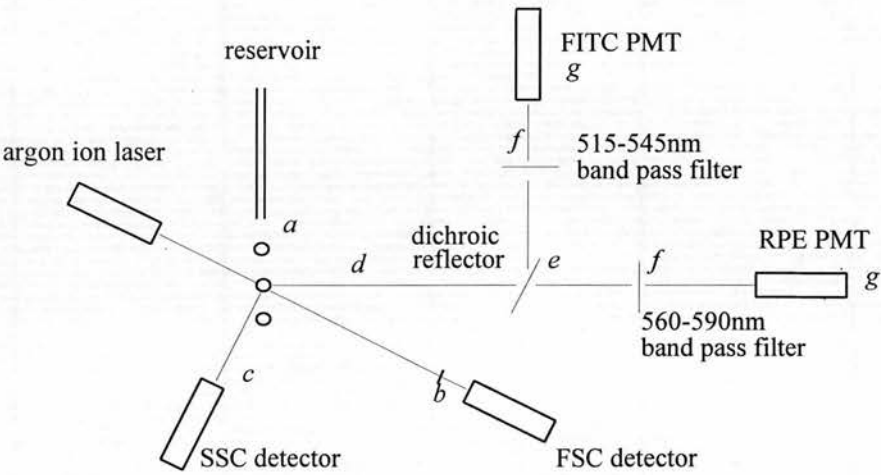
Fluorochrome dyes which absorb light at a specific wavelength and emit light at a higher wavelength have proved useful as detector systems. Fluorescein isothiocyanate (FITC) can be readily conjugated to mAbs without loss of antibody specificity or avidity, or dye quenching. It absorbs light at 488nm and emits at around 520nm. A group of natural fluorochromes, the phycobiliproteins (which include phycoerythrin (RPE), phycocyanin and allophycocyanin), are derived from red algae and photosynthetic cyanobacteria. RPE absorbs maximum light at 505nm and 75% of maximum at 488nm, emitting at 578nm. Because of their similar excitation wavelength but discrete emission wavelengths FITC and RPE can be used together in dual immunofluorescence studies. Initially, three colour immunocytometry was only possible using fluorochromes with higher excitation and emission wavelengths, necessitating the use of a dual laser instrument. Latterly, a number of tandem dyes have been developed for single laser three colour work (*e.g.* Red613 and Quantum Red) containing a donor fluorochrome (usually RPE) which emits light within the excitation wavelength of an acceptor fluorochrome, which emits light at a higher wavelength (*e.g.* Cy5 in Quantum Red) [Glazer & Stryer, 1983]. The combination emits light at a higher wavelength (630-670nm). Very recently a new dye, peridinin chlorophyll protein (PerCP) has been developed which absorbs light at 488nm and emits at 675nm, bringing the potential advantages of a simpler labelling protocol and less fluorescence overlap (Chapter 3.5.1).

Cellular autofluorescence, a product of normal cell constituents such as flavins

and cytochromes, provides the background against which a specific fluorescence label must be detected. Moreover, many cells express Fc gamma receptors which bind mAbs through the Fc arm of the antibody, leading to false interpretation of those cells as antigen positive. Where possible fluorochrome-conjugated antibody fragments (Fab or F(ab')<sub>2</sub>) adsorbed with human serum proteins were used in order to reduce non-specific uptake and cross-linking of antigenic sites which can precipitate cell aggregation. To achieve accurate information by immunofluorescence considerable care must be devoted to the choice of mAb and fluorochrome dye, optimal cell preparation and labelling technique.

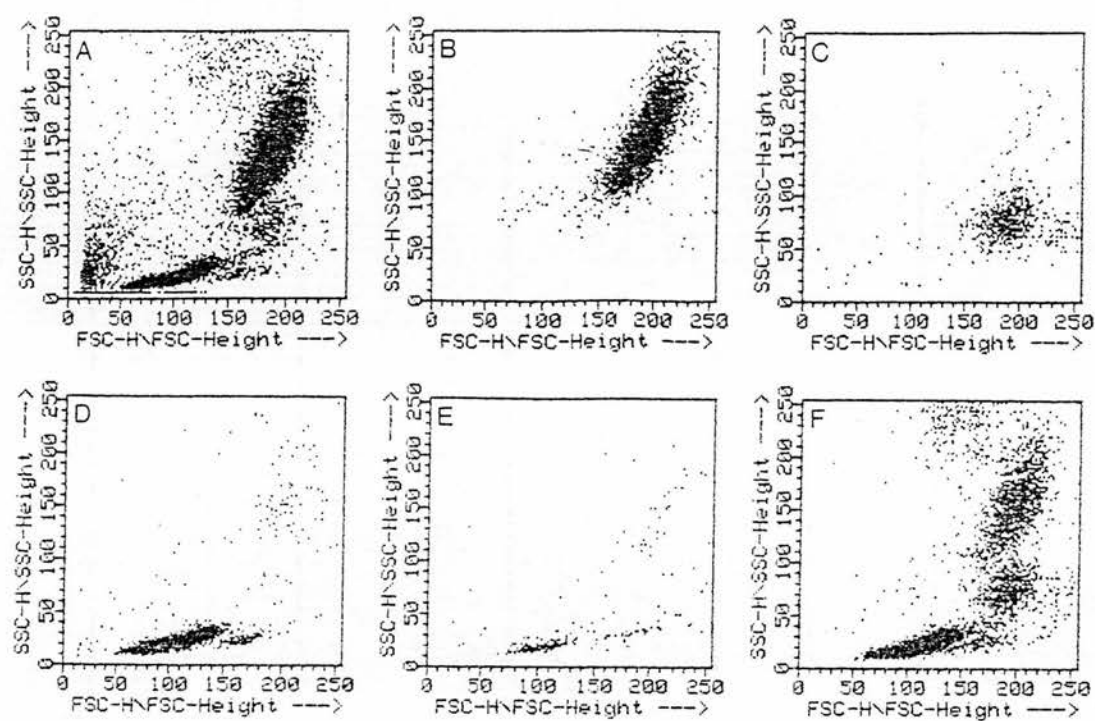
Flow cytometry provides multiparameter measurements on large numbers of cells in suspension, allowing characterisation of cell populations and the identification of component subpopulations. The fluorescence activated cell scanner (FACS) is equipped with an argon laser tuned at 488nm, power 14.8mW. It combines measurements of intrinsic cell properties (forward and side light scatter), with quantitative assessment of fluorescence [Parks, Lanier & Herzenberg, 1986]. Cells in suspension pass from a reservoir into the centre of a nozzle, where they are surrounded by cell free sheath fluid which confines the cells to the centre of a liquid jet. The laser emits light which is of single wavelength (monochromatic), in phase (coherent), and unidirectional with all rays parallel (collimated). As cells pass through the focused laser beam they scatter laser light, and fluorescent molecules that they contain or carry, are excited and fluoresce. A forward light scatter (FSC) detector detects light scattered at small angles from the axis of the laser, and converts it to an electronic signal. A side light scatter (SSC) detector detects light perpendicular to the axis. Fluorescent light is directed to separate photomultiplier detectors by a dichroic reflector. Optical filters reject scattered light, and help to define the acceptance wavelengths for each detector (Figure 2.2). Light scatter and fluorescence detector signals are amplified and digitised and stored by computer. Light scatter analysis provides information about relative cell size and internal complexity, which proves useful for discrimination of cell types in heterogeneous populations (Figure 2.3). Light scatter is most proportional to overall cell size at

**Figure 2.2 Schematic of the Optical Systems of a Fluorescence Activated Cell Scanner (FACS).**



*Legend.* Monochromatic collimated light (480nm) from an argon ion laser illuminates the stream of individual cells (a). Light reflected due to the size and granularity of the cell is detected by the forward (b) and orthogonal side (c) scatter detectors respectively. Cellular and membrane-bound fluorochromes absorb incident light and emit light at a higher wavelength (d). The dichroic reflector (e) reflects light of lower wavelength and allows light of higher wavelength to pass through. Further refinement is achieved by the band pass filters (f) which permit passage of a very restricted range of light wavelengths. The photomultiplier tubes (PMT) (g) convert light intensity into electrical voltage, which is then digitalised and analysed by computer.

Figure 2.3 FSC / SSC Characteristics of Mature Cell Populations.



*Legend.* Forward and side scatter characteristics of leukocyte populations in lysed peripheral blood: unlabelled (A); CD16<sup>+</sup> neutrophils and natural killer cells (B); CD14<sup>+</sup> monocytes (C); CD3<sup>+</sup> T lymphocytes (D); CD19<sup>+</sup> B lymphocytes (E); CD45<sup>+</sup> leucocytes (F).

smallest angles from the laser axis (FSC). At large angles (in the  $90^\circ$  range), light scatter is significantly affected by internal structure, granularity and nuclear shape (SSC). Back angles ( $180^\circ$ ) show weak signals, similar in informational content to the corresponding forward angles. Light scatter from non-spherical cells can be affected by cell orientation. The measurement of fluorescent light requires that several criteria be fulfilled, namely that fluorescence signals should be proportional to the amount of dye in or on the cell, that there should be sufficient signal for analysis and that there is a high signal to noise ratio. Signal / dye proportionality can be degraded if the laser illumination is not sufficiently high or if there is non-linearity in the electronics due to photomultiplier detector or amplifier saturation. Fluorescence signals from a particular dye are passed through a system of optical filters to discriminate against light from other sources - for example the exciting laser beam, stray room light, cellular autofluorescence or fluorescent light from other dyes. Since laser light is several orders of magnitude brighter than fluorescence, the filters must have an attenuation approaching 6 orders of magnitude at the laser wavelength. Optical filtering is achieved in two ways. Firstly by use of a dichroic reflector which reflects shorter wavelengths, whilst allowing longer wavelengths to pass. These are useful for separating signals when several dyes are excited by one laser. Secondly, by use of selected band pass filters which will only transmit light within a narrow band of wavelengths (usually in the order of 30nm) (see Figure 2.2). Photomultiplier detectors are used for low level fluorescence detection because they offer high gain / low noise amplification of the photoelectronic signal produced by the fluorescent light, and good linearity over a wide range of signal levels. The relationship between the intensity of detected light and the electronic signal generated is dependent on the voltage of photomultiplier detector (gain), and the mode of amplification. The choice between linear and logarithmic amplification depends on the range of signals to be measured, and the type of distribution expected. Linear amplification is appropriate for signals that vary over a small range, such as light scatter. Logarithmic amplification is particularly useful for immunofluorescence, where a single sample may contain



autofluorescent cells, stained cells several times as bright, and stained cells tens or hundreds as times as bright. Logarithmic amplification was used during the acquisition and visualisation of fluorescence data, but the data was converted to linear values by the software prior to mathematical analysis.

The detector voltages and signal amplification on the flow cytometer are adjusted for each series of experiments using negative control (irrelevant-specificity, isotype-specific) and positive control (anti-CD45) stained samples for each fluorescence parameter to be measured. The study population needs to be evenly distributed on the FSC / SSC dot-plot, and the negative control cell population should fall on the left of the fluorescence histograms. If the histogram is too far to the left, however, low fluorescence cells will summate in the 0 column and undermine the validity of the measurements of mean fluorescence intensity (mfi) *vide infra*. Spectral overlap occurs in systems in which more than one fluorescent dye is in use. Compensation is achieved by running positive control samples stained with one dye and adjusting the signal output from the complementary detector such that the histogram is the same as that of the negative control (Figure 2.4). This is carried out by adjustment of the linear amplifier (the photomultiplier detector voltage should remain constant).

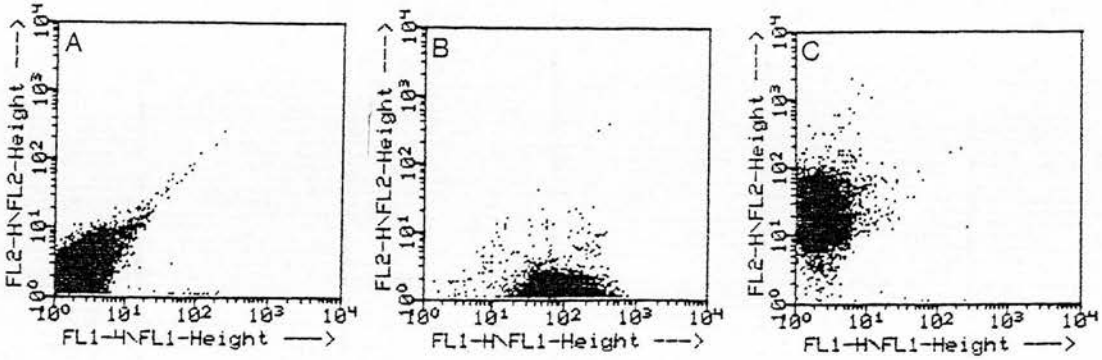
Once the cytometer was set-up for a particular series of experiments, the parameters were not changed, so that data were comparable between experiments.

#### 2.4.2 Titration and handling of monoclonal antibodies.

MAbs were purchased from a commercial source (Appendix 1), reconstituted according to manufacturers' instructions, aliquoted and stored frozen (-70°C) if non-conjugated, or at +4°C if conjugated. Prior to use mAbs were spun at 16,000g for 30min at +4°C using an Eppendorf centrifuge in a cold room, to remove aggregates. This manoeuvre was repeated on a weekly basis for the directly conjugated mAbs and sheep anti-mouse (SAM) FITC and RPE conjugates. Each mAb was titrated prior to routine use on an appropriate cell population. Table 2.2 shows the data from a representative experiment in which the anti-CD34 mAb 8G12-RPE was titrated on



Figure 2.4 Negative and Positive Controls and Compensation.



*Legend.* Negative and single stained CD45-FITC and CD45 SAM-RPE positive controls were used to establish satisfactory fluorescence gains and compensation.

(A) Negative control (B) CD45-FITC control (C) CD45 SAM-RPE control.

Compensation ensures that fluorescence in the unstained channel of the stained positive controls (*i.e.* FL-2 in B and FL-1 in C) is similar to that in the negative controls (*i.e.* A).

**Table 2.2 Titration of 8G12-RPE on KG1a.**

<i>Volume of 8G12-RPE</i>	<i>%fl*</i>	<i>mfi</i>	<i>RI</i>
20µl	98.7%	1181 ± 530	2.23
10µl*#	98.4%	1068 ± 504	2.12
5µl	98.7%	1076 ± 503	2.14
1µl	97.9%	618 ± 529	1.17
0.5µl	87.4%	176 ± 145	1.21
0.1µl	5.1%	25 ± 34	0.74
0.05µl	6.8%	30 ± 24	1.25
0.01µl	0.04%	3 ± 1	1.48
negative control	0.01%	1.2 ± 1.5	

*Legend.* A range of volumes of 8G12-RPE were used to label  $5 \times 10^5$  KG1a in order to establish the optimum titration for routine use. Percentage positive fluorescence (%fl\*), mean fluorescence intensity (mfi) and resolution index (RI) were calculated. The mAb may be used down to a concentration of 5µl /  $5 \times 10^5$  cells.

\* manufacturers recommendation. # volume used.

the KG1a cell line. A range of volumes of the mAb were used to stain  $5 \times 10^5$  KG1a, though equally serial dilutions of the same volume could have been used. In this case 5-10  $\mu$ l is adequate to achieve saturation of available epitopes. Below this, the mean fluorescence intensity (mfi) and the percentage positive fluorescence (%fl<sup>+</sup>) begin to fall because the quantity of mAb is inadequate. Above this the amount of non-specific binding and low-affinity Fc receptor uptake of mAb is increased.

The negative controls should be used at a similar volume and concentration to the test mAbs. This allows calculation of a resolution index (RI) for the mAb

defined as: 
$$RI = \frac{mfi+ - mfi-}{\sqrt{SD+^2 + SD-^2}}$$

where mfi+ and SD+ represent the mean fluorescence intensity and standard deviation of the positive population, and mfi- and SD- represent the mean fluorescence and standard deviation of the negative control population [Martini, D'Hautcourt, Brando *et al.* 1989].

The peak resolution index represents the optimal mAb concentration, because below this the mfi of the positive population will be low, and above this the mfi of the negative population will be high (due to non-specific uptake). The standard deviations give a measure of the distribution of fluorescence such that a mAb giving a consistent fluorescence intensity will have a higher RI than one with a similar mfi but more diffuse or patchy staining. The use of a standard deviation assumes a normal distribution of fluorescence which clearly does not pertain under most circumstances. It is often more convenient to titrate a mAb against a homogeneous cell line, but the target population may have a different level of antigen expression or level of Fc receptor uptake compared to the cell line, and hence the optimal titre may differ. Optimally, each batch of each mAb would be re-titrated against all possible targets. In practice, each batch of mAb was titrated against the most convenient target, *i.e.* cell line and / or leucapheresis product. The majority of commercial mAbs were found to lie within their optimal resolution indices at 10  $\mu$ l /  $5 \times 10^5$  cells - as recommended by the manufacturers. A standardised volume was used wherever possible.

#### 2.4.3 Optimisation of immunofluorescence labelling.

Human blood or bone marrow cells were enumerated using an automated counter, and aliquots containing  $5 \times 10^5$  leucocytes were used for each test and control sample. Cells were handled in a medium consisting of isosmotic phosphate buffered saline (PBS), containing 1% bovine serum albumin (BSA) to prevent cell death, 0.1% sodium azide to inhibit modulation and capping of surface antigens, and 0.02% EDTA to prevent cell clumping (*handling medium* Appendix 2). Test samples were routinely processed in parallel with negative control samples stained with mAbs of irrelevant specificity but of the same isotype as the experimental antibody. Wherever possible IgG1 mAbs were used, because IgG2a and IgM isotypes are reported to show higher non-specific binding and therefore a lower resolution index (*vide infra*). Directly conjugated, irrelevant-specificity mAbs were used as controls for directly conjugated mAbs, whilst purified irrelevant mAbs were used for indirect systems along with the appropriate second stage reagent. The routine test panel for single and dual colour labelling is outlined in Table 2.3.

The cells were washed twice in the handling medium, pelleted each time by centrifugation at 200g for 5min prior to removal of the supernatant. Preliminary tests showed that this centrifuge time and speed was sufficient to achieve a pellet, with negligible cell loss due to buoyancy or traumatic fragmentation. Cells were resuspended by gentle shaking by hand rather than vortex, again to minimise cell trauma. Cells were incubated with 0.5% solution of human gamma globulin in PBS for 15 minutes to reduce non-specific and Fc receptor-mediated uptake of antibody on the cell surface (*human gamma globulin solution* Appendix 2). Using the direct system, the cells were incubated with the primary murine mAb directly conjugated to a fluorochrome for 15 min. Using the indirect system, the cells were incubated with the primary murine mAb for 15 min, washed twice in handling medium, and incubated with a sheep anti-mouse antibody (SAM)-fluorochrome conjugate, again for 15 min. A further wash was carried out prior to red cell lysis and fixation. All washing, centrifugation and incubation was carried out at +4°C to reduce antigen capping.

**Table 2.3 Test Panels for CD34 Quantitation.**

#1: CD34 + SAM-FITC.

#2: CD45 + SAM-FITC.

#3: IgG1 + SAM-FITC.

#4: nil

*or*

#1: CD34-FITC

#2: CD45-FITC

#3: IgG1-FITC

#4: nil

*or*

#1: CD34-RPE + CD45-FITC.

#2: IgG1-RPE + IgG1-FITC.

*Legend.* CD34: purified mAb. SAM-FITC: sheep anti-mouse antibody conjugated to FITC. CD34-FITC: anti-CD34 mAb directly conjugated with FITC. The same abbreviations apply to the CD45 and IgG1 (negative control) mAbs, and the RPE conjugates. Within each test protocol the labelled samples were compared with similarly conjugated mAbs of the same isotype but irrelevant specificity.

---

Some authors have advocated shortening the preparatory procedure by omitting most of the washing steps [Caldwell & Taylor, 1986], pointing to advantages in preparation time and reduction in selective cell losses due to osmotic and traumatic fragility, and the higher buoyant density of activated and malignant cells and cell lines. This approach was not adopted because of the higher background fluorescence incurred, leading to higher thresholds on the negative controls and obscuration of small cell populations with low positive fluorescence.

#### *2.4.4 Effect of erythrocyte lysis and cell fixation on the physical and fluorescence characteristics of the study population.*

High erythrocyte contamination in the sample leads to a low leucocyte percentage in the acquired population and exacerbates the difficulty in studying the CD34<sup>+</sup> population. Two general approaches were taken to remove erythrocytes from the study population. Ficoll discontinuous density centrifugation is highly effective, but has been reported to lead to HPC loss [Wunder, Sovalat, Fritsch *et al.* 1992], an observation which was confirmed in a later study (Chapter 6.6.3).

As an alternative, erythrocytes were lysed by incubation with 0.5ml of a solution containing 8.29g/l ammonium chloride, 37mg/l EDTA and 0.84g/l sodium hydrogen carbonate in distilled water, adjusted to a pH of 7.3-7.5 using a pH meter [Hoffman & Hansen, 1981]. The osmolarity of the solution was checked using a Micro-Osmometer and found to be in the order of 298mOsm (*erythrocyte lysis solution* Appendix 2).

An experiment was carried out to determine whether erythrocyte lysis induced alteration in the physical or fluorescence characteristics of the study population. A sample of leucapheresis product (LP) was stained with CD34-RPE and CD45-FITC, and, along with the relevant negative controls, was subjected to no-lysis, a lysis wash, or 15, 30, 45 or 60 min exposure to the lysis solution at ambient temperature. The results are summarised in Table 2.4. The whole blood sample (no lysis) has been swamped with erythrocytes, leading to very low CD45<sup>+</sup> and CD34<sup>+</sup> percentages. On the LP, a threshold change in all parameters can be seen between no-lysis and lysis wash, corresponding to the elimination of contaminating erythrocytes (note that the CD45<sup>+</sup>% rises from 27% to 95% and CD34<sup>+</sup> from 0.6% to 2.1%). No changes occurred thereafter despite up to 1hr incubation. The conclusion is therefore that satisfactory erythrocyte lysis can be achieved with this reagent, without progressive alteration in physical or fluorescent characteristics of the leucocytes.

For standard purposes, the incubation in lysis solution was carried out for 5 min at ambient temperature, after immunofluorescence staining was completed. The



**Table 2.4 Effects of Erythrocyte Lysis on Physical and Fluorescence Characteristics of CD34 and CD45 Labelled Leucocytes.**

<i>Sample</i>	<i>FSC</i>	<i>SSC</i>	<i>FL-1</i>		<i>FL-2</i>	
			<i>IgG1</i>	<i>CD45</i>	<i>IgG1</i>	<i>CD34</i>
<i>whole blood</i>				3% (2)		0.1%(9)
<i>leucapheresis product</i>						
no lysis	98	34	0.05% (4)	27% (5)	0.05% (3)	0.6% (3)
wash	132	33	0.66% (9)	95% (15)	0.52% (10)	2.1% (10)
15min	131	42	0.69% (9)	94% (15)	0.46% (10)	2.5% (10)
30min	123	44	0.53% (7)	91% (13)	0.51% (7)	1.5% (7)
45min	128	42	0.82% (7)	95% (13)	0.62% (6)	2.4% (6)
60min	123	55	0.85% (6)	93% (15)	0.64% (7)	2.2% (7)

*Legend.* Results are expressed as the mean of 3 experiments. Physical characteristics are expressed as a mean value, fluorescence characteristics as % fluorescence positivity (%fl<sup>+</sup>) and as mean fluorescence intensity (in parenthesis). High levels of erythrocyte contamination lead to low CD34 and CD45 percentages in non-lysed whole blood and (to a lesser extent) leucapheresis product. No progression occurs in the physical or fluorescence characteristics of the study population with prolonged incubation in the lysis solution.

cells were washed in handling medium, and if red cell contamination was still judged to be too high, these steps were repeated. In practice, it was found that samples of LP or BM mononuclear cells required only a single lysis step, whilst whole blood or marrow often required two steps.

The flow cytometer was not on site, and therefore samples required fixation and storage in 2% paraformaldehyde prior to analysis. The distribution and intensity of immunofluorescence on human and murine cells fixed in paraformaldehyde is reported to be identical to that of fresh, unfixed cells. Cells can be stored at +4°C in the dark, for up to two weeks without difficulty, though after several weeks autofluorescence does increase [Lanier & Warner, 1981; Lal, Edison & Chused, 1988]. Samples of peripheral blood mononuclear cells were analysed with and without paraformaldehyde, and following 14 days storage (Table 2.5). Minor changes in the physical and fluorescence characteristics of the population occurred over this period, which could largely be compensated by adjustment of the cytometer. Samples were analysed within a few days of preparation where possible.

Some groups mix the test cells with propidium iodide prior to flow cytometry as a measure of viability. The stain is taken up by non-viable cells which fluoresce brightly in FL-2 and can be excluded during data acquisition. Unfortunately this was not a practical option because fixed cells stain uniformly with this dye. Fresh cells were used for staining wherever possible, and trypan blue dye exclusion was used to assess viability where necessary.

#### *2.4.5 Set-up of the flow cytometer.*

Samples were acquired and analysed using a FACScan flow cytometer (FACS), with an 488nm Argon ion laser emitting light at 14.7 millivolts. Optimal cytometry settings were established using a series of test samples, and maintained throughout the duration of the study unless otherwise indicated. The routine cytometer settings are detailed in Table 2.6.

For routine CD34<sup>+</sup> enumeration an acquisition gate was established on the FSC / SSC dot-plot by a process of back-gating on the CD45<sup>+</sup> control (Figure 2.5). In an

**Table 2.5 Effects of Paraformaldehyde Storage on Physical and Fluorescence Characteristics of CD34 and CD45 Labelled Cells.**

<i>Parameter</i>	<i>PBS</i>	<i>Para</i>	<i>Para day +14</i>	<i>cf: Para</i>	<i>cf: Para day +14</i>
FSC	102 ± 2	103 ± 2	96 ± 5	ns	p<0.05
SSC	69 ± 13	63 ± 12	59 ± 9	ns	p<0.05
FL-1 (CD45 <sup>+</sup> )	93.8 ± 0.7 (347 ± 55)	89.3 ± 3.0 (266 ± 71)	77.0 ± 23.0 (212 ± 46)	ns (ns)	ns (p<0.05)
FL-2 (CD34 <sup>+</sup> )	0.23 ± 0.13 (18 ± 5)	0.18 ± 0.10 (13 ± 2)	0.46 ± 0.59 (30 ± 4)	ns (ns)	ns (p<0.05)

*Legend.* Peripheral blood mononuclear cells prepared over Ficoll-Hypaque density gradient were labelled with CD34-RPE and CD45-FITC. Results are expressed as the mean ± standard deviation of 4 experiments. Physical characteristics are expressed as a mean value, fluorescence characteristics as percentage fluorescence positivity (%fl<sup>+</sup>) and as mean fluorescence intensity (in parenthesis). Results in PBS are compared to those in paraformaldehyde and to those after 14 days storage in paraformaldehyde by paired *t* test. Minor changes in the physical characteristics of the cells occurred which were easily compensated by adjusting the acquisition gate. A significantly lower FL-1 mfi and higher FL-2 mfi at day +14 was demonstrated. This was compensated during setting of the thresholds on the negative controls such that no significant difference in %fl<sup>+</sup> existed. Samples were analysed as soon after staining as reasonably possible.

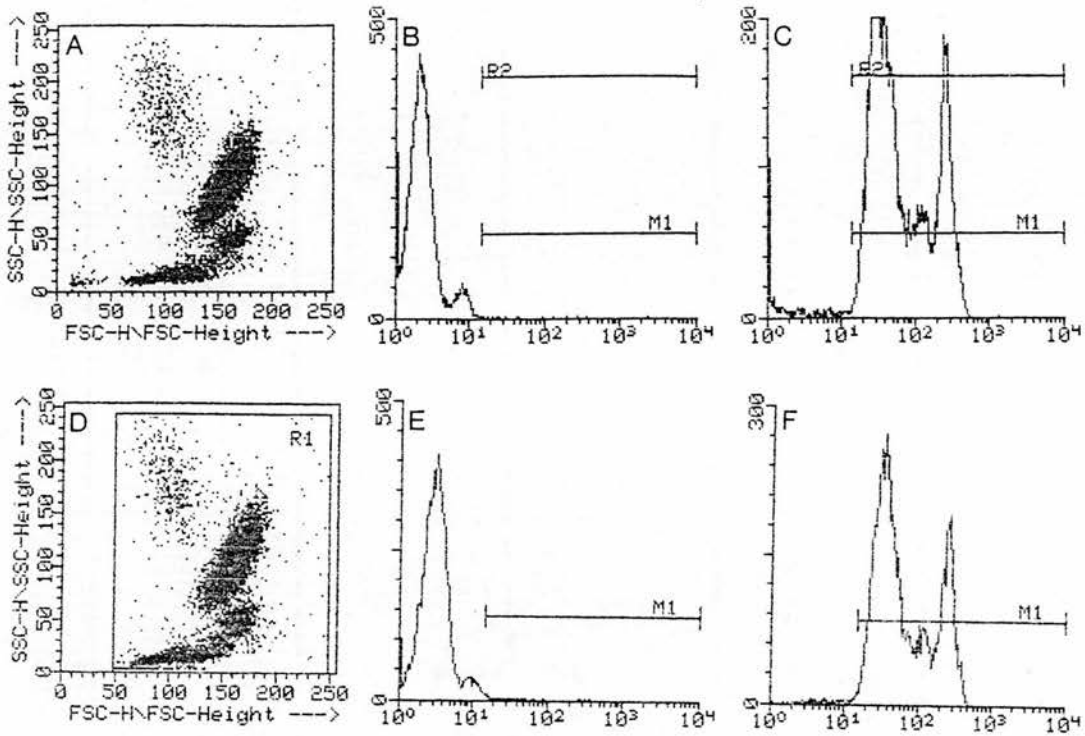
*PBS:* phosphate buffered saline. *Para:* paraformaldehyde.

**Table 2.6 Flow Cytometry Settings for Single and Dual Colour Immunocytometry of Leucocytes.**

<i>Parameter</i>	<i>Detector</i>	<i>Amplification</i>	<i>Threshold</i>
FSC	EOO	1.30	52
SSC	340	1.00	
FL1	575	Log	
FL2	521	Log	
<i>Compensation</i>			
FL-1 - %FL-2	1.7		
FL-2 - %FL-1	42.0		

*Legend.* Flow cytometer settings and compensation established for single and dual colour immunocytometry on positive and negative control samples. Once established, the settings were not changed during a series of experiments in order to maintain comparability of data.

**Figure 2.5 Backgating on the CD45<sup>+</sup> Population to Define Acquisition Gates for CD34<sup>+</sup> Enumeration.**



*Legend.* Lysed peripheral blood leukocytes acquired on an open gate (A) are contaminated by a variable number of residual erythrocytes and debris: negative control M1: 0.71% (B), CD45<sup>+</sup> M1: 92.54% (C). A gate (R2) established on the CD45<sup>+</sup> population allows these cells to be projected on the FSC / SSC dot-plot and an acquisition gate (R1) to be drawn such as to exclude most of the CD45<sup>+</sup> debris and high FSC cell aggregates (D). Negative control M1 on the R1 population is now 0.15% (E) and CD45<sup>+</sup> M1: 98.79% (F).

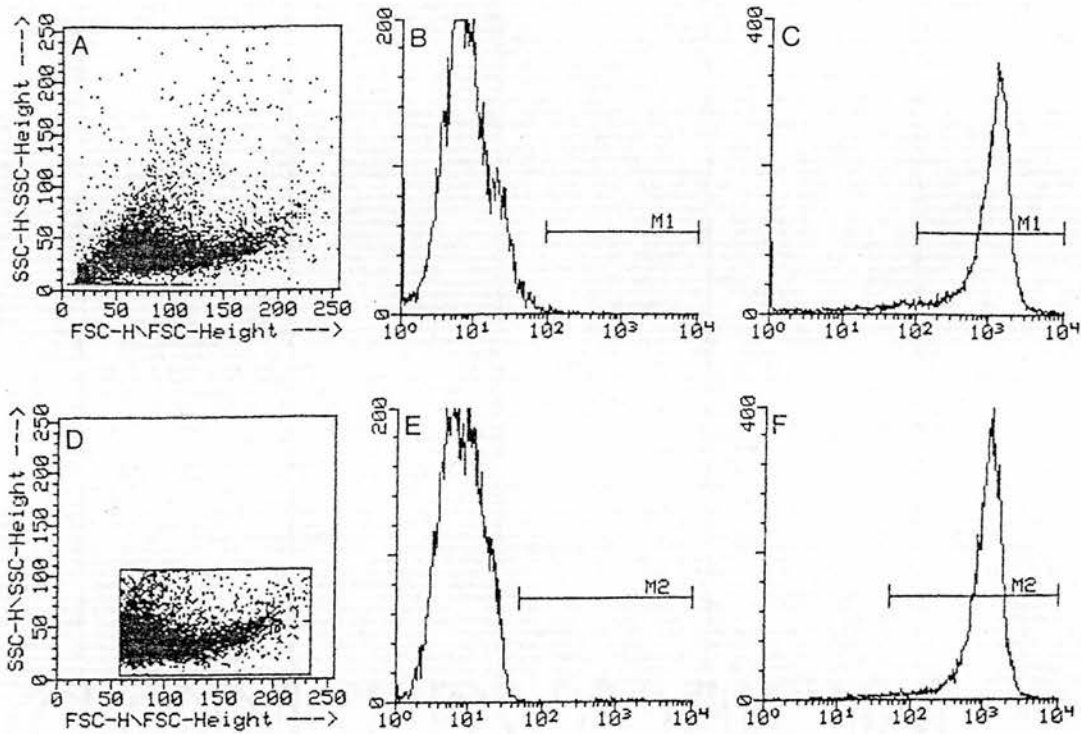
adaptation of the method described by Loken [Loken, Brosnan, Bach *et al.* 1990; Stelzer, Shults & Loken, 1993], negative and positive control cells were acquired ungated, and the CD45<sup>+</sup> population was defined on the appropriate fluorescence histogram (FL-1). The software program (Lysis II) was used to superimpose the CD45<sup>+</sup> cells on the FSC / SSC dot-plot, and an acquisition gate was set to exclude CD45<sup>-</sup> erythrocytes and debris, and cell aggregates. It is important when setting an acquisition gate to achieve the right balance between a gate which is too permissive - in which the leucocytes may be swamped by erythrocytes and debris, and too restrictive - in which one risks excluding CD34<sup>+</sup> cells with low FSC. The process of backgating allows one to place the acquisition gate more accurately, and to compensate for inter-sample variation. 10-50,000 events were acquired in list mode for each analysis. Data were analysed using the Lysis II software.

#### *2.4.6 Comparative study of the efficacy of available anti-CD34 monoclonal antibodies and direct / indirect systems of labelling.*

A comparative study was carried out of the efficacy of available anti-CD34 mAbs, in both indirect and direct labelling systems. The human myeloblastic cell line KG1a was used for this study, because the high constitutive expression of the CD34 antigen allows unimpeded analysis of the monoclonal antibodies [Civin, Strauss, Brovall *et al.* 1984]. Preliminary study revealed that KG1a were large cells, with high FSC and medium-high SSC profile, though a significant population of smaller cells / cell fragments were also present, presumably due to the persistence of effete and dead cells in the culture media and / or traumatic fragmentation during the preparatory procedure. The FSC amplification was reduced to 1.00 in order to bring the singlet population on scale, but other than this the flow cytometry settings did not need to be altered from those established on normal leucocytes (Table 2.6). Approximately  $87 \pm 3\%$  KG1a in healthy liquid culture expressed the CD34 antigen and backgating revealed that these were predominantly within the singlet cell population. Acquisition gates set around this population displayed a maximum CD34 expression of  $98 \pm 1\%$  with a mfi of  $1181 \pm 530$  (Figure 2.6, Tables 2.7 - 2.9).



Figure 2.6 CD34 -Labelling of KG1a With and Without Gating.



*Legend.* KG1a acquired on an open gate (A) include a significant amount of CD34<sup>-</sup> debris: negative control M1 0.2% (B), CD34<sup>+</sup> M1 91.5% (C). Acquisition on a narrow gate around the singlet population (D) reduces the negative tail (fluorescence summated in the 0 column): negative control M2 0.2% (E), CD34<sup>+</sup> M2 98.7% (F). If the culture was allowed to deteriorate the amount of CD34<sup>-</sup> debris increased.

**Table 2.7 Evaluation of anti-CD34 (FITC) Monoclonal Antibodies on KG1a.**

<i>antibody</i>	<i>%fl<sup>+</sup></i>	<i>mfi</i>	<i>RI</i>
QBEND10 SAM-FITC	99 ± 0.9%	1385 ± 214	1.46 ± 0.09
My10 SAM-FITC	98 ± 1.8%	1025 ± 59	1.30 ± 0.12
BI-3C5 SAM-FITC	87 ± 13.6%	561 ± 85	0.96 ± 0.02
TüK 3 SAM-FITC	94 ± 6.2%	425 ± 110	
12-8 SAM-FITC	97 ± 1.1%	1780 ± 374	1.10 ± 0.06
IgG1 SAM-FITC	0.5%	7 ± 2	
IgM SAM-FITC	0.5%	7 ± 2	
QBEND10-FITC	58 ± 11%	197 ± 44	1.74 ± 0.47
8G12-FITC	86 ± 5%	334 ± 13	1.80 ± 0.06
IgG1-FITC	0.5%	7 ± 3	

*Legend.* Results reported are the mean ± standard deviation of 3 replicate experiments. The best mAbs over all parameters were the IgG1 isotypes QBEND10 and My10, and the IgM 12-8 using an indirect sheep anti-mouse FITC conjugate (SAM-FITC). Purified 8G12 was not available for evaluation. The RI for TuK 3 could not be calculated because no IgG3 negative control was available. Direct conjugates QBEND10-FITC and 8G12-FITC demonstrated satisfactory RI but less %fl<sup>+</sup>.

*Abbreviations.* %fl<sup>+</sup> percentage fluorescence positivity. *mfi*. mean fluorescence intensity. *RI*. resolution index. SAM-FITC: sheep anti-mouse FITC conjugate.

**Table 2.8 Evaluation of anti-CD34 (RPE) Monoclonal Antibodies on KG1a.**

<i>antibody</i>	<i>%fl<sup>+</sup></i>	<i>mfi</i>	<i>RI</i>
QBEND 10 SAM-RPE	97 ± 1.9%	536 ± 337	1.18 ± 0.28
My10 SAM-RPE	89 ± 7%	356 ± 296	0.96 ± 0.24
BI-3C5 SAM-RPE	83 ± 10%	217 ± 80	0.82 ± 0.15
TüK 3 SAM-RPE	42 ± 17%	107 ± 96	
12-8 SAM-RPE	68 ± 22%	367 ± 509	0.46 ± 0.38
IgG1 SAM-RPE	0.5%	5 ± 3	
IgM SAM-RPE	0.5%	4 ± 3	
QBEND 10-RPE	3 ± 3%	61 ± 37	0.35 ± 0.11
8G12-RPE	99 ± 0.2%	1,108 ± 63	2.16 ± 0.06
IgG1-RPE	0.5%	1 ± 1	

*Legend.* Results reported are the mean ± standard deviation of 3 replicate experiments. The SAM-RPE indirect label was not as satisfactory as the SAM-FITC (Table 2.7). 8G12-RPE proved to offer a superior RI compared to the indirect labels.

*Abbreviations.* *%fl<sup>+</sup>* percentage fluorescence positivity. *mfi* mean fluorescence intensity. *RI* resolution index. SAM-RPE sheep anti-mouse RPE conjugate.

**Table 2.9 Evaluation of Negative Control Antibodies on KG1a.**

<i>FL-1</i>			<i>FL-2</i>		
<i>antibodies</i>	<i>%fl<sup>+</sup></i>	<i>mfi</i>	<i>antibodies</i>	<i>%fl<sup>+</sup></i>	<i>mfi</i>
auto	0.5 ± 0%	6.1 ± 1.3	auto	0.5 ± 0%	3.1 ± 0.9
IgG1 SAM-FITC	1.1 ± 0%	7.1 ± 2.2	IgG1 SAM-RPE	1.1 ± 0.5%	4.5 ± 2.8
IgM SAM-FITC	0.8 ± 0.3%	6.6 ± 2.3	IgM SAM-RPE	0.9 ± 0.7%	4.1 ± 2.7
IgG1-FITC	1.5 ± 0.6%	6.7 ± 3.2			

*Legend.* Data represents the mean ± standard deviation of 3 replicate experiments.

*Abbreviations.* *%fl<sup>+</sup>*: percentage fluorescence positivity. *mfi*: mean fluorescence intensity.

of  $149 \pm 3$  (within the CD34<sup>+</sup> population) of a representative normal human bone marrow aspirate (see Chapter 3.5.2).

Isotype-specific irrelevant mAbs and second step reagents were evaluated as negative controls against autofluorescent KG1a with a marker set at 0.5% ( $0.5 \times 10^6$  cells / test, 20  $\mu$ l neat mAb), and acquisition gates set around the main KG1a population as detailed above. The SAM-FITC and SAM-RPE were F(ab)<sub>2</sub> fragments in order to minimise uptake by Fc receptors, and were used at a titration of 1:20. The comparative %fl<sup>+</sup> and mfi of unstained (autofluorescent) cells and negative controls is documented in Table 2.9. The percentage of cells binding mAb in a non-specific manner (after subtraction of the autofluorescent population) can be summarised as: IgM 0.35% < IgG1 0.6% < IgG1-FITC 1.0%. Little difference was demonstrated between the use of SAM-FITC and SAM-RPE.

The currently available mAb to CD34 were evaluated for comparative efficacy by percentage of KG1a displaying positive fluorescence, mfi of the positive population and resolution index (RI) comparative to the appropriate isotype-specific negative control. No IgG3 control was available for Tük 3. Direct and indirect reagents were used as appropriate. 10,000 events were collected within an acquisition gate set around the singlet KG1a population. Results quoted are the mean of 3 replicate experiments (Tables 2.7, 2.8 & 2.9). The variation in the fluorescence characteristics of the KG1a (expressed as the standard deviation, SD), is a reflection of variability in experimental technique, in particular in cellular autofluorescence and nonspecific mAb binding, and in cell size and acquisition gates. The best mAbs over all parameters (Tables 2.7 & 2.8) were the IgG1 isotypes QBEND10 and My10, and the IgM 12-8. Purified 8G12 was not available for evaluation. Using an indirect technique and SAM-FITC up to 98% of gated KG1a were CD34<sup>+</sup>, with an mfi of >1000, and resolution indices greater than 1.3. BI-3C5 and Tük 3 proved inferior by most criteria, though a correct negative control for Tük 3 was not available and the RI was therefore not calculated. Indirect labelling with SAM-RPE proved inferior to SAM-FITC with almost all mAbs. The direct conjugates 8G12-FITC and QBEND10-FITC, proved inferior in terms of %fl<sup>+</sup> and

mfi, but superior in terms of RI. 8G12-FITC was superior to QBEND10-FITC by all criteria (Table 2.7). 8G12-RPE became available in mid-1993, and proved equivalent to the indirect labels in terms of %<sup>+</sup> and mfi, but markedly superior in terms of RI (Table 2.8). The superior resolution with direct conjugates is explained by the reduced intra-experimental variability in fluorescence (*i.e.* the standard deviation of the mfi) allowing greater discrimination between positive and negative populations.

The optimal immunofluorescence labelling protocol for CD34 quantitation drawn from the studies detailed in this section is summarised in Table 2.10 and the test panel noted in Table 2.3. This protocol is similar to that recommended by the 1st and 2nd European workshops on HPC determination [Wunder, Sovalat, Fritsch *et al.* 1992; Wunder, Sovalat Henon *et al.* 1994].

## **2.5 Determination of the Resolution Limits of Immunocytometry.**

It was clearly desirable to attempt to determine the resolution limits of the immunocytometry technique which are dependent on the accuracy of the flow cytometer, variation in the properties of the cells and an acceptable coefficient of variation (cv).

In a preliminary experiment, a peripheral blood mononuclear preparation (unstained) was put through the flow cytometer in a series of 10 acquisitions of 10,000 events, and a further 10 acquisitions of 50,000 events. On the 10,000 event acquisition, a marker set at 0.5% positive fluorescence demonstrated an actual %fl<sup>+</sup> of  $0.45 \pm 0.06\%$  (cv=12.9%), and a marker set at 0.05% demonstrated an actual %fl<sup>+</sup> of  $0.04 \pm 0.02\%$  (cv=39.9%) (Results quoted as mean  $\pm$  standard deviation of 10 replicate experiments). Similar markers set on the 50,000 event acquisition demonstrated actual %fl<sup>+</sup> of  $0.50 \pm 0.06\%$  (cv=12.1%) and  $0.04 \pm 0.03\%$  (cv=96.3%) respectively. The cv using a higher %fl<sup>+</sup> marker was clearly more satisfactory, though there was no significant difference between 10 and 50,000 event acquisitions (p=ns, non-paired *t* test).



**Table 2.10 Synopsis of the Labelling Protocol for Single Colour Immunocytometry.**

5x10<sup>5</sup> cells / test.

washed twice in 0.5ml of handling medium.

incubated for 15min in 0.5% human gamma globulin solution.

incubated for 15min with 10µl mAb (See Table 2.3).

washed twice in handling medium\*.

incubated for 15min with 10µl 2<sup>nd</sup> step reagent\*.

washed once in handling medium.

incubated for 5-10min in erythrocyte lysis solution (until clarification)<sup>s</sup>

washed once in handling medium<sup>s</sup>.

resuspended in 0.5ml paraformaldehyde.

*Legend.* The formulation of the solutions used is described in the text and in Appendix 2. Reagents were maintained and all centrifugation, incubation and storage carried out at +4°C. \*indirect labelling techniques only. <sup>s</sup>omitted for cell lines, samples prepared over Ficoll-Hypaque and some leucapheresis products.

A series of experiments was carried out using serial dilution of KG1a in peripheral blood MNC in order to establish correlation between expected CD34<sup>+</sup> counts and measured counts, and to establish the range of the cv - especially at the lower limits of resolution. Peripheral blood MNC were spiked with KG1a at a range of relevant concentrations, and were labelled with QBEND10 and SAM-FITC using standard technique. Cells were acquired ungated, and analysed relative to a 0.5% marker established on the FL-1 histogram of non-spiked (but labelled) PB-MNC. The results are presented in Table 2.11. The correlation coefficient between predicted and measured CD34%<sup>+</sup> was highly significant ( $r=0.98$ ,  $p<0.001$ ). As can be seen, the KG1a population was 86.4% CD34<sup>+</sup> and a CD34<sup>+</sup> KG1a% corrected for this factor is quoted in column 2 of Table 2.11. There was a persistent shortfall in the proportion of CD34<sup>+</sup> KG1a detected compared to that predicted, though whether this was due to a technical artefact such as inaccuracy in the dilution technique, or to interaction between KG1a and the mononuclear cell population, was unclear. The cv lies between 3 and 15% over a wide range of concentrations, until the %fl<sup>+</sup> falls below 1% where the cv deteriorates.

## **2.6 Evaluation of Different Approaches to Data Analysis.**

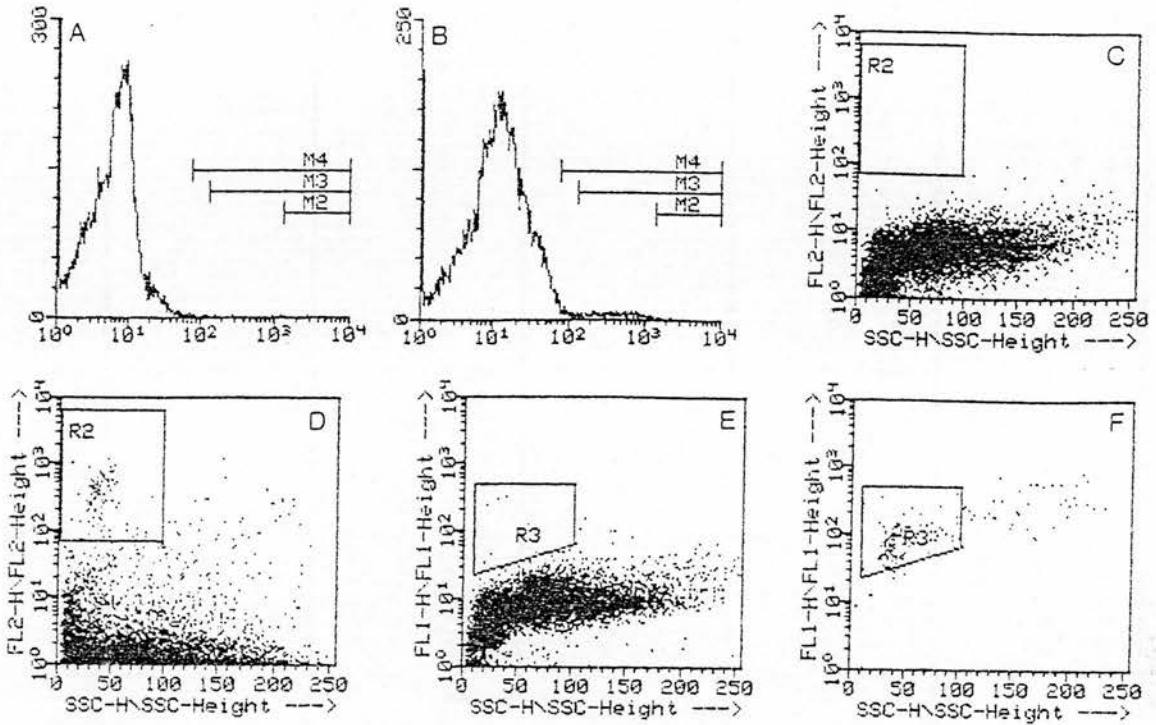
An experiment was carried out to establish the optimum method of quantitating the CD34<sup>+</sup> population within a given sample. PB and LP samples were studied from 10 patients undergoing mobilisation with cyclophosphamide and rhuG-CSF. Each sample was labelled with 8G12-RPE and CD45-FITC, with appropriate negative controls. 30-50,000 events were acquired through a permissive gate established on the CD45<sup>+</sup> backgated FSC / SSC dot-plot, set to exclude debris and aggregates only. Although this leads to a lower overall CD45%<sup>+</sup> than a more restrictive gate, it prevents exclusion of small CD34<sup>+</sup> cells. Data was analysed using three markers on the negative control FL-2 histogram, 0.01%, 0.05%, and a marker at the point of minimum inflection (Figure 2.7). Regions were established on the correlative dot-plot of SSC / FL2, to include 0.01-0.05% of the low side scatter cells of the negative control. In addition, the CD34<sup>+</sup> population could be backgated onto the correlative

**Table 2.11 Serial Dilution of KG1a in Peripheral Blood Mononuclear Cells.**

<i>KG1a dilution</i>	<i>corrected CD34%<sup>+</sup></i>	<i>observed CD34%<sup>+</sup> ± SD (cv)</i>
100%	86.4%	86.4 ± 11.1% (12.8%)
75%	64.8%	50.1 ± 1.7% (3.4%)
50%	43.2%	30.9 ± 3.0% (9.6%)
25%	21.6%	12.1 ± 1.7% (14.0%)
12.5%	14.5%	6.5 ± 0.7% (10.8%)
6.25%	7.23%	3.3 ± 0.5% (14.7%)
3.12%	2.69%	1.8 ± 0.1% (3.4%)
1.56%	1.35%	1.1 ± 0.1% (5.3%)
0.78%	0.67%	0.6 ± 0.15% (26.3%)
0.39%	0.34%	0.5 ± 0.00%
0.195%	0.168%	0.47 ± 0.15% (31.9%)
0.00%	0.00%	0.47 ± 0.06% (12.8%)

*Legend.* In the second column the CD34%<sup>+</sup> is corrected for the fact that 86.4% of KG1a are CD34<sup>+</sup>. Results in the third column are the mean ± standard deviation of 3 replicate experiments. The coefficient of variation is quoted in parentheses. The coefficient of variation was satisfactory above approximately 1% CD34<sup>+</sup>.

Figure 2.7 Comparative Approaches to CD34 Enumeration.



*Legend.* Several approaches to enumeration of the CD34<sup>+</sup> population were examined. Markers established on the FL-2 negative control (A) at M2 0.01%, M3 0.05% and M4 (point of minimal inflection) 0.1%, revealed CD34<sup>+</sup> of 0.04%, 2.21% and 2.34% respectively (B). A region established on the SSC / FL-2 dot-plot demonstrated a negative control (R2) of 0.01% (C) and a CD34<sup>+</sup> of 2.12% (D). Backgating the CD34<sup>+</sup> population (defined on M4) onto the SSC / FL-1 dot-plot demonstrates that the CD34<sup>+</sup> cells are predominantly CD45<sup>lo</sup> and SSC<sup>lo</sup>: negative control (R3) 0.02% (E), CD34<sup>+</sup> 2.00% (F).

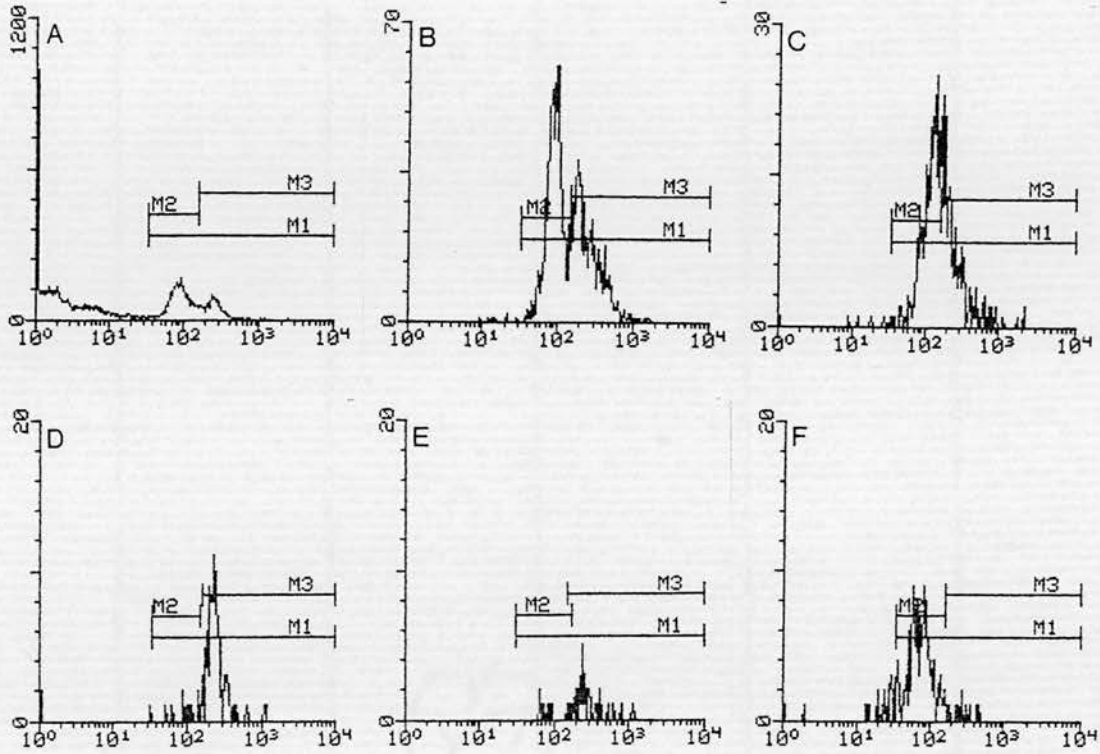
dot plot of SSC / FL-1 (CD45) , and a region established to include 0.01-0.05% of the CD45<sup>-</sup> SSC<sup>lo</sup> cells on the negative control.

Paired *t* tests were carried out to establish whether significant differences existed between the CD34<sup>+</sup> counts determined by different analytical methods. The CD34<sup>+</sup> counts determined on the histograms were significantly different such that those calculated on a 0.01% marker were less than those calculated on a 0.05% marker ( $p < 0.001$ ), which in their turn were less than those calculated on a variable marker ( $p < 0.001$ ). Clearly, some CD34<sup>+</sup> cells have low fluorescence, are obscured by the background population, and are ignored when a stringent analytical marker is employed. The background fluorescence achieved on the dot-plots was less than that on the 0.05% histogram, but there was no significant difference between the two dot-plot analyses, indicating that the exclusion of SSC<sup>hi</sup> cells was the critical factor, and that CD45<sup>-</sup> cells do not contribute significantly to the background. There was no significant difference between the CD34%<sup>+</sup> on the 0.05% histogram and the 2 dot-plot analyses.

In conclusion there is probably no unequivocally "correct" method of establishing positive CD34 fluorescence. A variable marker on the histogram gives a higher overall CD34<sup>+</sup> count due to the inclusion of CD34<sup>lo</sup> cells, but produces a high background and is difficult to standardise. Although there was no difference between the 0.05% histogram marker and the two dot-plots, the latter display a lower false positive background. Use of a very high marker (0.01%) on the histogram alone, leads to underestimation of the CD34 count. Several authors have argued that true CD34<sup>+</sup> cells show low side scatter, and are CD45<sup>int</sup>, and that these characteristics can be used to discriminate true CD34<sup>+</sup> cells from false uptake by neutrophils (SSC<sup>hi</sup>, CD45<sup>int</sup> or CD45<sup>hi</sup>), monocytes (SSC<sup>int-hi</sup>, CD45<sup>hi</sup>) and lymphocytes (SSC<sup>lo</sup>, CD45<sup>hi</sup>) [Trischmann, Schepers & Civin, 1993; Sutherland, Keating, Nayar *et al.* 1994]. A proportion of CD34<sup>+</sup> cells are CD45<sup>hi</sup> (Figure 2.8), and there seems no reason *a priori* to dismiss these as false positive staining lymphocytes. In fact, no particular advantage to backgating the CD34<sup>+</sup> population onto a SSC / FL-1 dot-plot prior to quantitation was demonstrated in this study. The



**Figure 2.8 CD45<sup>+</sup> Fluorescence of Various Populations of Leucocytes.**



*Legend.* Leucocyte populations were dual stained with Lin SAM-RPE and CD45-FITC. (A) All cells; (B) CD16<sup>+</sup> gated neutrophils and NK cells; (C) CD14<sup>+</sup> gated monocytes; (D) CD3<sup>+</sup> gated T lymphocytes; (E) CD19<sup>+</sup> gated B lymphocytes; (F) CD34<sup>+</sup> gated HPC. Although all leucocytes are >97.5% CD45<sup>+</sup> (M1), they can be subdivided into CD45<sup>hi</sup> and CD45<sup>lo</sup> populations (M2 & M3) (A). T and B lymphocytes fall predominantly into a CD45<sup>hi</sup> subpopulation (M3) (D & E), whilst neutrophils and monocytes demonstrate a broad spectrum of CD45 fluorescence (B & C). HPC are predominantly CD45<sup>lo</sup> (M2) (F). Several groups have recommended exclusion of CD45<sup>hi</sup> SSC<sup>hi</sup> cells during CD34 enumeration, though overlap in the properties of different populations does occur and this strategy does lead to ignorance of a CD34<sup>+</sup> subset.



more contentious issue as to whether CD34<sup>+</sup> high side scattering cells should be dismissed as false positive is returned to in Section 2.9.3 and Chapter 3.5.4.

The variable contamination of the study sample with erythrocytes presents a problem in achieving accurate CD34<sup>+</sup> enumeration. Samples were routinely run in tandem, *i.e.* one stained with anti-CD34 and anti-CD45 mAbs, and the other with the appropriate negative controls. The measured CD34%<sup>+</sup> was corrected for erythrocyte contamination by division with the CD45%<sup>+</sup>

$$i.e. \quad CD34\%^{\circ} = \frac{CD34\% - Neg\ control\%^{+}}{CD45\%^{+} - Neg\ control\%^{+}} \times 100$$

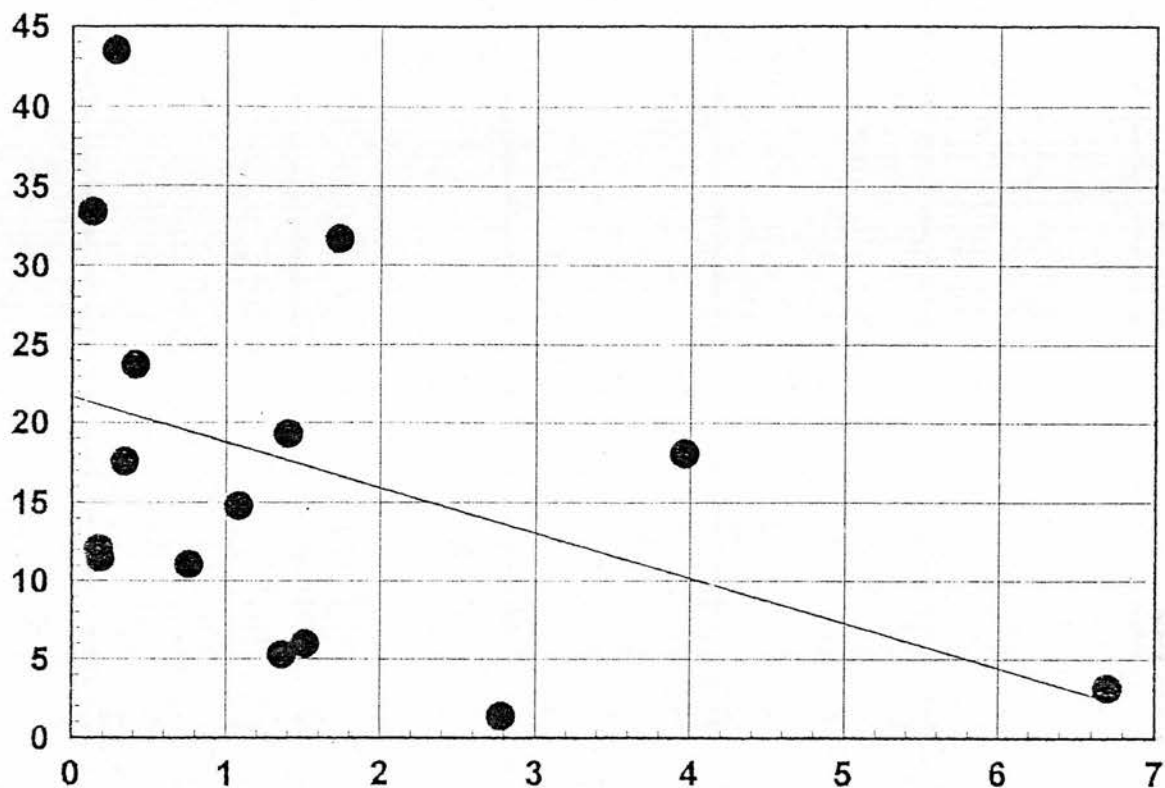
The absolute number of CD34<sup>+</sup> cells in the sample (CD34<sup>n</sup>) can be calculated by interpreting the CD34%<sup>+</sup> as a percentage of the absolute leucocyte count measured by a cell counter. This calculation is dependent on the assumption that all leukocytes are CD45<sup>+</sup>. Nucleated erythroid precursors are CD45<sup>-</sup>, and although the Coulter ZF and T-890 instruments were satisfactory for analysing peripheral blood samples, microscopic examination of the film was required to obtain a differential count of nucleated erythrocytes from other nucleated cells in bone marrow samples.

## 2.7 Study of the Overall Accuracy of CD34 Quantitation.

In order to establish the overall accuracy with which CD34<sup>+</sup> populations can be defined and quantitated a series of experiments was carried out in which samples of BM, PB<sup>m</sup>, LP and CB were stained in four separate experiments with 8G12-RPE and CD45-FITC using standard labelling protocol, acquired through a CD45-backgated FSC / SSC gate, and analysed on the basis of overall CD45 positivity and CD34 positivity on the FL-2 / SSC dot plot.

The mean CD34%<sup>+</sup>, SD and cv of the four replicate experiments / sample were calculated for a total of 15 samples, the results of which are presented in Figure 2.9. The median CD34%<sup>+</sup> was 1.08% (range 0.14 - 6.70) and the median cv 14.8% (range 1.4 - 43.5). There was a weak negative correlation between CD34%<sup>+</sup> and cv ( $r = -0.42$ ) with a regression line described by the formula:  $y = 21.2 - 2.86.x$ . The

Figure 2.9 Overall Accuracy of CD34<sup>+</sup> Quantitation.



*Legend.* CD34%<sup>+</sup> was analysed on 4 aliquots from each of 15 samples from BM, PB<sup>m</sup>, LP and CB and the mean, standard deviation and coefficient of variation of the replicate assays was calculated for each sample. There was a weak negative correlation between CD34%<sup>+</sup> and cv ( $r = -0.42$ ) with a regression line described by the formula:  $y = 21.2 - 2.86.x$ . *Abscissa:* CD34%<sup>+</sup>, *Ordinate:* coefficient of variation. Solid data points are observed results. The results are tabulated in Appendix 3 Table A.1.

bone marrow samples demonstrated a CD34%<sup>c</sup> of  $0.63 \pm 0.64\%$  (n=4), PB<sup>m</sup> of  $2.26 \pm 2.20\%$  (n=8) and CB of  $0.69 \pm 0.52\%$  (n=4).

## **2.8 Establishment of a Correlation between CD34<sup>+</sup> and CFU-GM Assays.**

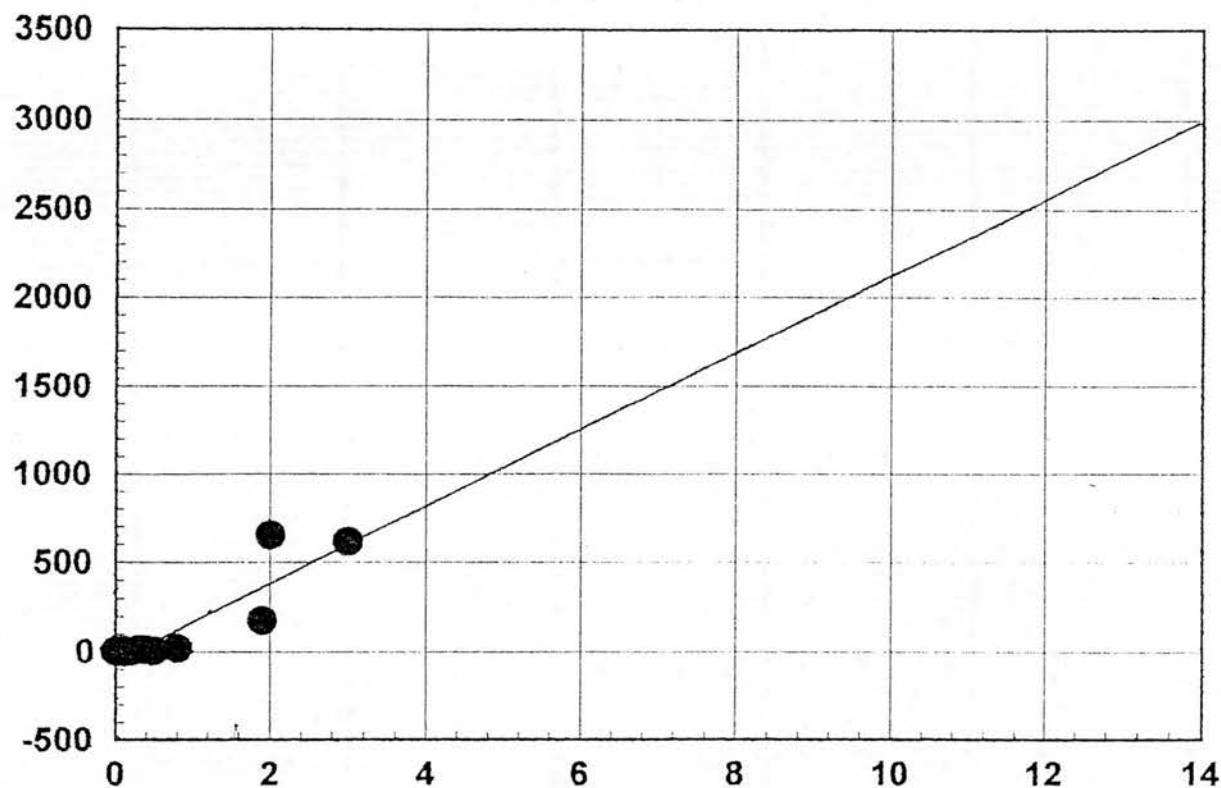
A direct correlation has been reported between the concentration of CD34<sup>+</sup> cells and CFU-GM in normal human marrow, and in mobilised peripheral blood [Fritsch, Emminger, Buchinger *et al.* 1991; Serke, Säuberlich & Huhn, 1991]. The CFU-GM cloning efficiency (CE) of an HPC population is defined as the percentage of CD34<sup>+</sup> cells giving rise to colonies [Wunder, Sovalat, Fritsch *et al.* 1992]

$$\text{i.e.} \quad \text{CFU-GM CE} = \frac{\text{number of CFU-GM (>50 cells)}}{\text{number of CD34}^+ \text{ cells}} \times 100$$

A series of 14 LP samples were analysed by CFU-GM and CD34 immunocytometry in order to assess whether the two assays provided comparable results. The data are presented in Figure 2.10, and give a correlation coefficient of  $r = +0.90$ , with a regression line formularised as  $y = -41.69 + 217.77.x$ . The cloning efficiency varies substantially from 0.4 to 32.5%. The correlation degrades below approximately 0.5% CD34<sup>+</sup> or 10 CFU-GM/10<sup>5</sup> cells. In fact, when the 10 samples in this subgroup were analysed separately, no correlation could be demonstrated ( $r=+0.06$ ).

Similarly, no correlation could be demonstrated between CD34 and CFU-GM in 10 samples from the blood of normal individuals ( $r= -0.11$ ) (Table A3).

**Figure 2.10 Correlation between CFU-GM and CD34<sup>+</sup> Cells in 14 Leucapheresis Product Samples.**



*Legend.* CD34%<sup>+</sup> and CFU-GM were analysed from 14 samples of LP. There is a strong positive correlation coefficient of  $r = +0.90$  with the regression line formularised as  $y = - 41.69 + 217.77.x$ , but this degrades below approximately 0.5% CD34<sup>+</sup> or 10 CFU-GM/10<sup>5</sup> cells ( $r = +0.06$ ). *Abscissa:* CD34%<sup>+</sup>, *Ordinate:* CFU-GM/10<sup>5</sup> leucocytes. Solid data points are observed results. Some data points overlap at the lower end of the range. The results are tabulated in Appendix 3 Table A.2.

## 2.9 Discussion.

The objective of the initial phase of this study was to establish an accurate and reproducible method with which to define the haematopoietic progenitor cell population and to validate the optimised immunocytometry system through comparison with an alternative (clonogenic) assay system and by collaboration with other research groups (*vide infra*).

### 2.9.1 Standardisation and reproducibility of the CFU-GM assay.

There are a number of factors which contribute to the difficulty in achieving standardisation and reproducibility of semi-solid clonogenic assays, and in achieving comparability of data between sources.

Firstly, cell survival and proliferation is dependent on the culture environment, including base medium, serum source, cytokines, humidity, oxygen and carbon dioxide tension (and pH), and presence of infection. Clonogenic assays are particularly dependent on the nature of the serum source and cytokine stimulation. There is considerable batch-to-batch variation in colony growth in FCS, though whether this is a reflection of the protein / lipid content, cytokine content or other factors, is unclear. Culture conditions can be standardised by use of commercial culture media (such as those marketed by Gibco BRL, Paisley or by Stem Cell Inc., Vancouver), or by the use of serum-free culture conditions and defined cytokine cocktails.

Secondly, the number and dispersion of colonies in the assay may be an important source of error during enumeration. The accuracy of clonogenic assays is fundamentally limited by the statistical accuracy inherent in counting a small number of colonies. In a group of  $x$  assays with a total of  $n$  colonies, the mean number of colonies per assay is  $n/x$  with a standard deviation  $\sqrt{n/x}$  due to the Poisson distribution [Blackett, 1974]. Therefore in a triplicate assay with a total of 3 colonies the SD is 0.58 and the cv is 58%, with a total of 30 colonies the SD is 1.83 and the cv is 18.25% and with a total of 300 colonies the SD is 5.77 and the cv is 5.8%. In addition to inherent statistical variation, considerable variation is added by

the observer. Accurate colony enumeration must be carried out carefully by a dedicated observer. A highly cellular background, clumping of nucleated cells, erythrocyte contamination or infection, can all give rise to considerable "noise" against which it proves impossible to enumerate colonies. Inadequate dispersion of cells in the culture media can lead to clumps or swathes of cells which may be mistaken for colonies. In addition, colonies can occur at any depth within the culture matrix (1-2mm) and may be missed if outwith the plane of focus. The formation of very high numbers of colonies ( $>1\text{-}200/\text{assay}$ ) or very low numbers ( $<10/\text{assay}$ ) is particularly problematic due to colony overlap and observer fatigue in the first instance and the high coefficient of variation in the second. A range of assays at different cell concentrations may need to be established.

Finally, the nature of the cell population under investigation may be an important determinant of the cloning efficiency of the HPC population. Not all  $\text{CD}34^+$  cells form colonies, as can be seen from the data in Section 2.8. There are several reasons for this. Not all  $\text{CD}34^+$  cells are necessarily HPC, for example in bone marrow  $\text{CD}34^+$  endothelial cells may be present. In addition, only a proportion of HPC form colonies under a given set of conditions. Colony formation is a composite product of HPC proliferation, differentiation and survival, and the cloning efficiency is as much a function of the culture conditions as it is of the HPC present. One can talk (for example) about the CFU-GM cloning efficiency and the BFU-E cloning efficiency of a given population, and although it is assumed that the cells which form CFU-GM under a given set of culture conditions are different to those which form BFU-E under a different set of conditions, this is not necessarily so. Adding to the complexity, Gordon *et al* [Lewis, Blackett & Gordon, 1994] have shown temporal succession of CFU-GM colony formation such that CFU-GM present at day +7 are mainly different colonies from those present at day +14 or at +21. Furthermore, it is likely that cloning efficiency is source dependent *i.e.* varies with the proportion of myeloid progenitors in the  $\text{CD}34^+$  population, and the presence and activation of accessory cells. Positive and negative cytokines are not only provided from exogenous sources (such as serum and conditioned medium),



but also from T lymphocytes and monocytes. The numbers and activation of accessory cells may vary substantially dependent on the source and *in vivo* cytokine exposure of the study population. In addition, the exogenous cytokines may act on accessory cells, leading to a secondary effect on HPC proliferation *in vitro* [Wunder, Bacrenzung, Sovalat *et al.* 1992]. Comparability of clonogenic assays between different source materials may therefore only really be achievable by the study of highly purified CD34<sup>+</sup> populations, and even then is only informative about a subset of the HPC population.

### 2.9.2 Standardisation and reproducibility of CD34<sup>+</sup> quantitation.

Two factors are of critical importance to the accurate resolution of HPC using CD34 immunocytometry, the choice of mAb-fluorochrome labelling system and adequate control over non-specific binding. My10, 8G12 and QBEND10 are the best current mAbs to the CD34 antigen. In the main, an indirect labelling system with SAM-FITC gives rise to brighter fluorescence than the direct conjugates, but at the cost of higher background fluorescence. 8G12-RPE proved satisfactory in terms of fluorescence intensity, with a low background and high resolution index allowing superior resolution of the CD34<sup>+</sup> population (Section 2.4.6). These results are broadly comparable with those published by the groups in Vancouver [Lansdorp, Sutherland & Eaves, 1990] and Milan [Ravagnani, Siena, Bregni *et al.* 1991; Siena, Bregni, Brando *et al.* 1991b]. Prior to 1992, QBEND10 was acceptable for indirect labelling, and was the only CD34 mAb available in a FITC-conjugated form. 8G12-FITC became available in this country in April 1992, and 8G12-RPE in mid 1993, and the latter was used in later studies. The optimised labelling and analytical protocol (Table 2.10) was not dissimilar to that arrived at by others [Siena, Bregni, Gianni *et al.* 1993; Siena, Bregni, Di Nicola *et al.* 1994; Bender, Unverzagt & Walker, 1994; Säuberlich, Kirsch & Serke, 1994; Gee & Lamb, 1994; Roscoe, Rybka, Winkelstein *et al.* 1994]. Detailed attention to technique is important to the validity of the results. The correlation demonstrated between CD34%<sup>+</sup> and CFU-GM (Section 2.8) is similar to that reported by several other groups [Siena, Bregni,

Brando *et al.* 1991a; Serke, Säubelich & Huhn, 1991; Bender, Williams, Myers *et al.* 1992; Fritsch, Buchinger & Printz, 1993c; Bender, Lum, Unverzagt *et al.*, 1994]. Many groups now use CD34 quantitation as a real-time marker for assessing the viability of a harvest, or to monitor peripheral blood stem HPC rebound following chemotherapy [Siena, Bregni, Belli *et al.* 1992].

Caution needs to be exercised in that the accuracy and reproducibility of CD34<sup>+</sup> counts remains dependent on careful methodology, and degrades at low percentages of CD34 positivity (Section 2.7). The correlation between CD34 positivity and CFU-GM also deteriorates below a level of about 0.5% CD34<sup>+</sup> (Section 2.8).

Data was contributed to two European workshops on HPC quantitation held in Mulhouse, France in 1992 and 1993 [Wunder, Sovalat, Fritsch *et al.*, 1992; Wunder, Sovalat, Hénon *et al.*, 1994], which attempted to achieve a consensus between field workers on optimal techniques for CD34 quantitation for both clinical and research use. As a study in standardisation, 22 European clinical and research laboratories conducted an experiment in which samples of a LP harvested from a normal adult volunteer in Berlin were assayed by each laboratory, using aliquots of the same staining reagents, and a commonly agreed technical and analytical protocol. Flow cytometers from five different companies were used, and cytometer set-up and acquisition gates could not be standardised. The percentage of non-viable cells as determined by dye-exclusion varied between 0 and 42%. False positive rates on the negative controls varied between 0.01 and 0.5%. Results obtained with the CD34-FITC conjugates were highly variable, with CD34<sup>+</sup> percentages determined by 8G12-FITC ranging from 0.00 to 1.29%, and those determined by QBEND10-FITC ranging from 0.17 to 17.8% with 5 failures. Results obtained in Berlin immediately after harvest revealed a percentage CD34<sup>+</sup> of 0.24 (8G12-RPE, mean of a quadruplicate determination on 50,000 cells). In summary, even a highly motivated group of experienced cytometry laboratories with an agreed protocol have difficulty establishing reproducibility [Sovalat, Wunder, Zimmermann *et al.*, 1994]. This is perhaps not particularly suprising in view of the high cv associated with replicate experiments in a single laboratory (Section 2.7).

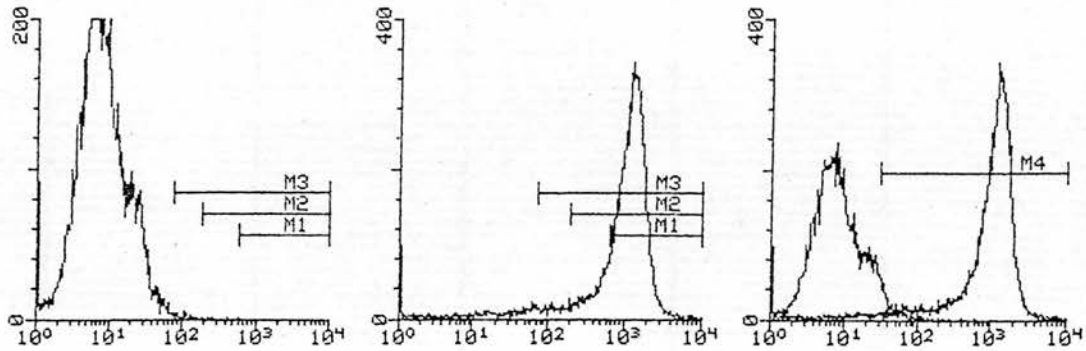
### 2.9.3 Theoretical considerations in the definition of CD34<sup>+</sup> populations.

The definition of positive fluorescence is such a fundamental issue in the interpretation of CD34 immunocytometry data, that it is discussed in some depth here. Negative control samples are established using the study sample, irrelevant-specificity isotype-specific mAbs, and identical technique. There are several schools of thought as to how the criteria for discrimination of positive and negative populations should be established [Martini, D'Hautcourt, Brando *et al.* 1990]. The first advocates that a marker should be set either at the point at which the number of false positives on the negative control equals the number of false negatives on the positive control (the point of zero differential), or just after the peak on the negative control, (the point of minimal inflection) (Figure 2.11). These approaches lead to a variable background level of false positive cells, which (particularly in the former) may be up to 20-25%. The advantage of this kind of approach is that it allows low intensity stained cells in the test sample to be included in the analysis.

The second school of thought sets a marker at a fixed percentage of false positive cells on the negative control (Figure 2.11), classically at 0.5-2%, - though much more stringent criteria can be used. This has the advantage that the extent of contamination of the positive population with false positives is quantifiable, but has the disadvantages that low intensity staining positive cells are operationally considered negative and that a few highly fluorescent cells in the negative control can cause a significant shift in the discriminatory marker to the right (the problem is compounded because the flow cytometer determines fluorescence on a cell by cell basis, rather than presenting a fluorescence profile of the whole population). The use of a discriminatory region on a fluorescence / SSC dot plot as discussed in Section 2.6 eases the problem since most false positive cells are high SSC neutrophils and monocytes, but is based on the assumption that CD34<sup>+</sup> HPC are never high scattering.

The third school of thought eschews the definition of positive and negative populations of cells in favour of direct comparison of mean fluorescence of negative control and labelled populations. This is very helpful where the study population is

**Figure 2.11 Methods of Discrimination of Positive and Negative Populations.**



*Legend.* Unstained (A) and CD34-RPE labelled (B) KG1a are used for illustration. Several methods may be used to discriminate fluorescence positive and negative populations. Fixed markers can be established at various levels of stringency on the negative control (A) M1 0.00%, M2 0.05%, leading to CD34<sup>+</sup> (B) of M1 77.55% or M2 88.88% respectively. Variable markers can be established at the point of minimal inflection M3 (A) 0.38% (B) 92.47% or at the point of zero differential on the overlay histogram (C) where the percentage false<sup>+</sup> on the negative control (M4 4.33%) roughly equals the percentage false<sup>+</sup> on the labelled sample (M4 95.22%). Where (as here) there is a homogeneous fluorescence positive population (as averse to generation of a single or complex subpopulations), the mean fluorescence intensity (A 11, B 1102) or the peak channel number (modal fluorescence) (A 8, B 1197) may prove a more useful measure.



more or less homogeneous (*e.g.* a cell line), but may be inappropriate where discrete or complex populations exist. A small shift in the mean fluorescence of a homogeneous population may be impossible to discriminate from a larger shift in the fluorescence of a discrete subpopulation, without direct inspection of the histogram. There are particular problems with the use of the standard deviation, which assumes a normal distribution of data around the mean. This is valid only under strictly defined circumstances, namely a cell population which is homogeneous in its expression of the antigen. The standard deviation was found to be useful in titration and comparative assessment of mAbs against cell lines, where a resolution index was calculated (Section 2.4.2 and 2.4.6).

These problems are particularly acute when studying small cell populations with low density antigen expression - such as CD34<sup>+</sup> HPC.

In this study the criteria employed to define the CD34<sup>+</sup> population and measure fluorescence within it, has been adapted to the purpose in hand. In defining the CD34<sup>+</sup> population for enumeration, the priority was seen as including most potential CD34<sup>+</sup> cells even at the cost of a higher false positive background (which can then be subtracted), *i.e.* high sensitivity with lower specificity. CD34 / CD45 stained cells were therefore acquired through a permissive gate and quantified on a 0.05% histogram marker or FL-2 / SSC dot plot as described above (Section 2.6). The risk of ignoring CD34<sup>+</sup> SSC<sup>hi</sup> cells on the latter was considered to be offset by the possible detection of CD34<sup>lo</sup> cells.

During 2 or 3 colour immunocytometry (Chapter 3) or FACS-based enrichment (Chapter 6.6), the priority is to achieve minimal contamination of the study population with false positive cells, even at the price of ignoring CD34<sup>lo</sup> cells (*i.e.* high specificity but low sensitivity). The threshold markers need to be highly *discriminatory* because when one comes to analyse the dual fluorescence data it is not possible to separate the true positive population from the false positive because the dispersal of the false positive fluorescence intensity is unknown. The problem is most acute when small levels of true positivity are under study. An increase in the false positivity rate (by lowering the discriminatory threshold) leads to increased

contamination of the positive population, especially at low percentage true positivity. If, for example, the negative control marker was set at 0.5% positive, and the test sample was 1% positive, one could deduce that 0.5% of the cells in the test sample were true positive, but one could not ascertain which these were. If, on dual immunofluorescence studies, the 1% apparent positive cells (0.5% false and 0.5% true) show 50% positive expression of a second antigen, it becomes impossible to deduce whether all the true positive cells (and none of the false positive) are coexpressing the second antigen, or none of the true positive but all of the false positive, or some proportion of each. Cells for dual colour immunofluorescence studies were acquired through a fairly restricted lymphoblastoid gate, judged by backgating the CD34<sup>+</sup> population onto the FSC / SSC dot plot (Chapters 3.2 and 3.3). In some of the studies using triple colour immunofluorescence, cells were acquired through a gate established on the FSC / FL-2 dot plot (Chapter 3.5). Here, a control acquisition was carried out through an open gate to provide an estimate of the contamination of the study population with false positive events. It should be reiterated that use of stringent criteria such as these biases the data in favour of more primitive, CD34<sup>hi</sup> HPC.

In the circumstance of analysing overall expression of an antigen on a cell line, or coexpression within a defined CD34<sup>+</sup> population, a less stringent *indicative* threshold marker of positivity (such as the point of minimal inflection), and / or the mean fluorescence intensity of the study population were found to be more useful (Chapter 3). This is because low intensity fluorescence may be missed by highly stringent markers, and sensitivity is of much more importance than specificity.

A final problem is the question of whether the false positive percentage should be subtracted from the actual positive percentage to reveal the "true positive" percentage. In my opinion the answer is no, because the term "false positive" assumes that these highly fluorescent cells are antigen negative, when in fact these are highly autofluorescent cells or those to which mAb has become inappropriately bound, and their antigenic status is indeterminate. In a population of cells in which 99% are CD44<sup>+</sup>, for example, it is unjustifiable to assume that a 5% "false positive"



population are CD44<sup>+</sup>. If the false positive population are a random selection of the background population, then the same proportion of these cells would be fluorescence positive as in the general population, and one could correct for the error. However, the assumption is not necessarily valid. In the analyses that follow, the actual measured percentage positivity is quoted, along with the percentage positivity in the negative controls. The use of discriminatory or indicative markers is stated overtly. Where appropriate, a two-tailed paired or non-paired *t* test has been carried out on the results of a series of experiments to compare a particular percentage positivity (%fl<sup>+</sup>) or mean fluorescence intensity (mfi) with negative controls or a parallel series of experiments respectively (discussed further in Chapter 3.2.1).

This extended analysis focuses on the issue of what is meant by the statement that particular cell or cell population is "positive". A final point should be emphasised: that fluorescence positivity referenced to an indicative or discriminatory threshold marker, cannot be interpreted as synonymous with the presence or absence of a given antigen on the cell surface. It is to be hoped that the correlation between antigen density and fluorescence intensity is linear, and the relationship may be quantitated by a reference titration based on standardised beads (Chapter 3.5.2). However, a given cell expressing the antigen at low density can quite clearly fall within the fluorescence negative population, especially if it starts from a low base (*i.e.* has lower than average autofluorescence).

This somewhat complex combination of approaches, then, was found to be most informative in analysing fluorescence structure in Chapter 3, where the initial objective was to examine cell adhesion molecule expression within CD34<sup>+</sup> populations from different sources.

## **CHAPTER 3. CHARACTERISATION OF HAEMATOPOIETIC PROGENITOR PHENOTYPE.**

### **3.1 Introduction. p115**

### **3.2 A Comparative Study of Haematopoietic Progenitor Cell Adhesion**

#### **Molecule Expression. p 116**

- .1 Study design and general methodology. p 116.*
- .2 Analysis of normal peripheral blood samples at rest and following exercise.*
- .3 Analysis of samples from normal bone marrow, mobilised peripheral blood and umbilical cord blood. p 122.*
- .4 Effects of mouse serum on binding of second-step reagent. p 127.*
- .5 Comparability of data and methodological problems. p 127.*

### **3.3 A Comparative Study of Haematopoietic Progenitor Expression of Activation and Lineage Markers. p 130**

- .1 Modifications in dual immunocytochemistry methodology. p 131.*
- .2 Results. p 132.*

### **3.4 Characterisation of Cell Adhesion Molecule, Lineage and Activation Marker Expression by Haematopoietic Cell Lines. p 140**

### **3.5 Development of Three-Colour Immunocytochemistry to Study Cell Adhesion Molecule Expression within Haematopoietic Progenitor Subsets. p 148**

- .1 Evaluation of potential third colour reagents. p 150.*
- .2 Use of calibration beads to assess the intensity of antigen expression. p 150.*
- .3 Contamination of the study population with variation in the discriminatory marker. p 155.*
- .4 Discrimination of CD34<sup>+</sup> lineage-subsets by light-scatter. p 161.*
- .5 Three colour analysis of adhesion molecule expression within bone marrow HPC subsets. p 163.*

### **3.6 Discussion. p 165**

- .1 Problems associated with the interpretation of adhesion molecule expression.*
- .2 Problems associated with the definition of HPC-subsets. p 171.*
- .3 Problems with three-colour immunocytochemistry of HPC subsets. p 174.*

### 3.1 Introduction.

The development of satisfactory immunocytometry methodology detailed in Chapter 2 permitted the use of multicolour immunocytometry for phenotypic haematopoietic progenitor (HPC) analyses. In an initial study of HPC cell adhesion molecule (CAM) expression, HPC were labelled with the directly conjugated anti-CD34 mAb QBEND10-FITC and cell adhesion molecules by purified mAbs with a sheep anti-murine R-phycoerythrin (SAM-RPE) second step reagent. FL-1 discriminators were set at a 0.5% false positivity rate and samples of less than 1% measured positivity (0.5% true positivity) were not studied on the grounds that more than 50% of the study population would not, in fact, be HPC (Chapter 2.9.3). This initial study suggested that significant differences do exist between sessile and circulating HPC (Section 3.2), but also that methodological weaknesses needed attention. The biological significance of the observations remained unclear for several reasons. In particular, it was uncertain whether differences in CAM expression reflected differences in the heterogeneity of the CD34<sup>+</sup> population or the effects of supraphysiological cytokine exposure, and whether phenotypic differences could be directly translated into differences in functional binding behaviour. It was therefore decided to study the pattern of lineage and activation markers in HPC derived from different sources (Section 3.3). Between 1992 and 1993 8G12-RPE became available and the superior resolution index of this mAb-conjugate (Chapter 2.4.6 and 2.9.2), along with improvements in the staining and analytical techniques, allowed a reduction in the false positive rate to 0.05% of the negative control. The implications in terms of improved precision, accuracy and comparability of the results are discussed in Section 3.3. The question as to whether different HPC subsets express different patterns of CAM was addressed first using a representative panel of human haematopoietic cell lines (Section 3.4), and later through development of three-colour immunocytometry (Section 3.5). The latter represented a considerable methodological challenge in view of the small numbers of cells under examination. The composite results of this series of experiments is discussed in Section 3.6. The development of an *in vitro* assay with which to study functional cell binding is discussed in Chapters 4-6.

## 3.2 A Comparative Study of Haematopoietic Progenitor Cell Adhesion

### Molecule Expression.

A dual immunocytometry study was designed to look at the coexpression of the CD34 antigen and a panel of cell adhesion molecules demonstrated by other groups to be expressed by bone marrow HPC, or which were thought likely to be expressed on the basis of known bone marrow stromal and endothelial structure (Table 3.1) [Gordon, 1988a & b; Soligo, Schiro, Luksch *et al.* 1990; Kansas, Muirhead & Dailey, 1990; Lewinsohn, Nagler, Ginzton *et al.* 1990; Long & Dixit, 1990; Arkin, Naprstek, Guarini *et al.* 1991; Papayannopoulou & Brice, 1992; Clark, Gallagher & Dexter, 1992; Saeland, Duvert, Caux *et al.* 1992; Simmons, Niutta, Ashman *et al.* 1992; Greenwalt Lipsly, Ockenhouse *et al.* 1992; Teixidó, Hemler, Greenberger *et al.* 1992; Liesveld, Winslow, Frediani *et al.* 1993; Lund-Johansen & Terstappen, 1993; Kobayashi, Imamura, Uede *et al.* 1994].

### 3.2.1 Study design and general methodology.

Five groups of samples were studied: peripheral blood from normal volunteers (n=12) (PB), bone marrow from normal volunteers (n=8) (BM); normal umbilical cord blood (n=8) (CB), and peripheral blood from patients following 4g/m<sup>2</sup> cyclophosphamide and 5µg/kg rhuG-CSF (n=8) (PB<sup>m</sup>), taken at two time points during the recovery phase following hypoplasia: as the peripheral blood leucocyte count rose above 1x10<sup>9</sup>/l (PB<sup>m</sup>#1), and 48hr later (PB<sup>m</sup>#2), corresponding to the rise and fall of circulating progenitors in the peripheral blood [Craig, Anthony, Stewart *et al.* 1993]. These sources are discussed in Section 2.2.1.

Dual immunofluorescence was carried out using a technique similar to that used for labelling cells with a single fluorochrome. Samples were prepared by discontinuous density centrifugation over Ficoll Hypaque. Mononuclear cells from the interface were collected, counted, and the viability assessed by trypan blue exclusion. Cells were washed thrice in handling medium, and 5x10<sup>5</sup> cells/test were incubated with rabbit serum for 15 min (to block Fc receptor uptake of mAbs) and re-washed. They were incubated for 30 min with 10µl of a pretitred purified mAb addressed to a cell adhesion

**Table 3.1. Cell Adhesion Molecules Studied.**

<i>Cell adhesion molecule (CD)</i>	<i>Ligand</i>	<i>mAb</i>
<i>Immunoglobulin Gene Superfamily.</i>		
ICAM-1 (CD54)	LFA-1, Mac-1	84HIO Immunotech
PECAM-1 (CD31)	PECAM-1 heparan sulphate	5.6E Immunotech
LFA-3 (CD58)	LFA-2	AICD58 Immunotech
<i>Integrin Family.</i>		
<i><math>\beta_1</math> VLA subfamily.</i>		
VLA-4 ( $\alpha^4\beta_1$ ) (CD49d/CD29)	Fn VCAM-1	HP2/1 Immunotech
VLA-5 ( $\alpha^5\beta_1$ ) (CD49e/CD29)	Fn	SAM1 Immunotech
<i><math>\beta_2</math> leukocyte adhesion subfamily</i>		
LFA-1 ( $\alpha^L\beta_2$ ) (CD11a/CD18)	ICAM1,2+3	IOT16 Immunotech
<i><math>\beta_3</math> cytoadhesion subfamily.</i>		
Vitronectin receptor ( $\alpha_V\beta_3$ ) (CD51/CD61)	vitronectin Fn, fibrinogen	AMF7 Immunotech
<i>Selectin Family.</i>		
L-Selectin (CD62L)	GlyCAM-1, MAdCAM-1, CD15, CD15s,	Dreg56 Immunotech
<i>Proteoglycan Analogues.</i>		
HCAM(CD44)	collagen hyaluronic acid	F10-44-2 Serotec
<i>CD36/LIMP II Family.</i>		
Thrombospondin receptor (CD36)	collagen thrombospondin	FA6-152 Immunotech



molecule (Table 3.1). After incubation the cells were washed thrice, and incubated with SAM- RPE pre-titred to 1/40, for 30 min. The cells were again washed thrice, incubated for 15 min with mouse serum to block uptake of the secondary mAb by unbound active sites on the SAM. The mouse serum was removed, and the cells washed and incubated for 30 min with 10µl of QBEND10-FITC. The cells were washed and resuspended in 1% paraformaldehyde. Positive controls were established using purified and FITC-conjugated mAbs addressed to CD45. Negative controls were established using irrelevant isotype-specific purified and conjugated mAbs as appropriate. All incubation, washing, centrifugation and storage, was carried out at +4°C. The routine method for dual immunofluorescence labelling is documented in Table 3.2, and the test and control panel in Table 3.3.

Compensation was set in the two fluorescence channels using the positive controls as previously detailed in Chapter 2.4.1 and Figure 2.4. Data acquisition was carried out with Consort 30 Data Management System. For clinical samples, 10,000 cells were acquired through a lymphoblastoid acquisition gate set to include cells of medium-high FSC and low SSC. This window has been shown to include the vast majority of colony-forming cells (CFU-GEMM, CFU-GM and BFU-E) and long term culture - initiating cells (LTC-IC) [Andrews, Singer & Bernstein, 1989]. The gate reduced background autofluorescence of myeloid cells and partially enriched for HPC, permitting better definition of the CD34<sup>+</sup> population [Civin, Banquerigo, Strauss *et al.* 1987]. FSC, SSC and two fluorescence signals were determined for each cell and stored in list mode data files. The analysis of the four dimensional data was performed with Consort 30 software. Discriminatory markers were established using the negative controls at 0.5% on the FL-1 and at the point of minimal inflection on the FL-2 histogram. A dual parameter contour map displaying FL-1 against FL-2 was used to define four cell populations, namely CD34<sup>-</sup> CAM<sup>+</sup> (#1), CD34<sup>+</sup> CAM<sup>+</sup> (#2), CD34<sup>-</sup> CAM<sup>-</sup> (#3) and CD34<sup>+</sup> CAM<sup>-</sup> (#4) (Figure 3.1). The percentage of events in quadrants 2 and 4 defined the percentage of CD34<sup>+</sup> HPC in the acquired sample, and allowed a statement of the percentage of CD34<sup>+</sup> cells coexpressing the CAM of interest. The arithmetic mean fluorescence intensity (mfi) of the CAM, SAM- RPE on the CD34<sup>+</sup> population was



**Table 3.2. Dual Immunofluorescence Labelling for the Cell Adhesion Molecule Study.**

low density mononuclear cells ( $5 \times 10^5$  cells / test)  
wash thrice in 0.5ml handling medium  
incubated with 20 $\mu$ l rabbit serum for 15min (Fc receptor blockade)  
wash once in 0.5ml handling medium  
incubated with 10 $\mu$ l of purified anti-CAM mAb for 30min  
washed thrice in handling medium  
incubated with 10 $\mu$ l of SAM-RPE for 30min  
washed thrice in handling medium  
incubated with 20 $\mu$ l of mouse serum for 15min  
wash once in handling medium  
incubated with 10 $\mu$ l of QBEND10-FITC for 30min  
washed once in handling medium  
resuspended in 1% paraformaldehyde

**Table 3.3. Test Panel for the Cell Adhesion Molecule Study.**

1. CD54	SAM-RPE	QBEND10-FITC
2. CD31	SAM-RPE	QBEND10-FITC
3. CD58	SAM-RPE	QBEND10-FITC
4. CD49d	SAM-RPE	QBEND10-FITC
5. CD49e	SAM-RPE	QBEND10-FITC
6. CD11a	SAM-RPE	QBEND10-FITC
7. CD51	SAM-RPE	QBEND10-FITC
8. CD62L	SAM-RPE	QBEND10-FITC
10. CD36	SAM-RPE	QBEND10-FITC
11. CD45	SAM-RPE	
12.		CD45-FITC
13. IgG1	SAM-RPE	
14. IgG2a	SAM-RPE	
15.		IgG1-FITC
16. Autofluorescent		

*Legend.* QBEND10-FITC: anti-CD34 mAb directly conjugated to FITC.  
CD\*\*: purified mAb addressed to the appropriate antigen. SAM-RPE: sheep anti-mouse antibody conjugated to RPE. The labelled samples were compared with similarly conjugated mAbs of the same isotype but irrelevant specificity.

calculated by summing RPE fluorescence intensity of the two CD34<sup>+</sup> quadrants.

On the assumption that the percentage positive fluorescence (%fl<sup>+</sup>) or the mean fluorescence intensity (mfi) of CD34<sup>+</sup> expression of a particular CAM would be normally distributed continuous variables over a series of experiments, results were quoted as mean  $\pm$  standard deviation (SD) of several replicate experiments (n). A two-tailed paired Student's *t* test was used to assess whether %fl<sup>+</sup> or mfi was significantly different from control, and a two-tailed non-paired *t* test was used to assess the significance of differences between sources (Table 3.4). The *p* value so derived provides a measure of the probability of obtaining the observed data if the null hypothesis is true *i.e.* if there is (in fact) no difference in the fluorescence of the two populations under comparison. The *t* tests have not been formally modified to correct for the effects of multiple comparisons [Altman, 1994], but in view of the fact that four sets of comparisons were intended for each CAM, the cut-off for statistical significance was considered to be  $p < 0.01$ .

### 3.2.2 Analysis of normal peripheral blood samples at rest, and following exercise.

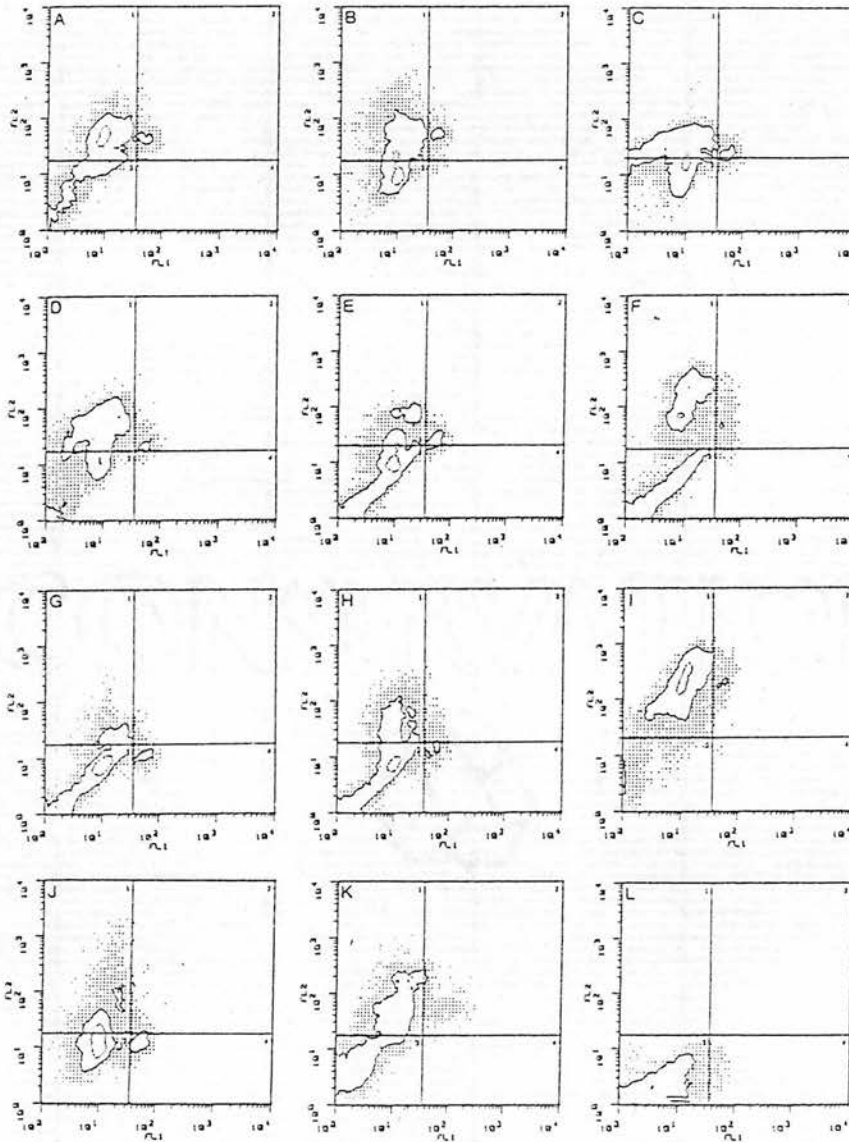
Peripheral blood samples were taken from normal male and female volunteers, prior to exercise (steady state), and immediately following vigorous exercise. Vigorous exercise is known to transiently mobilise HPC, probably by displacing them from a margined population. The volunteers exercised on a static bike for 10 minutes, at 30km/h and maximum tolerated work load. Five men and five women (median age 31.5; range 25-38) achieved a median heart rate of 155bpm (range 130-180), which represented 70-95% of maximal heart rate (calculated as 220-age). A  $1.5 \pm 0.2$  fold increase in peripheral blood leucocytes occurred. CD34<sup>+</sup> levels were below threshold levels prior to ( $0.28 \pm 0.17$ ), and following ( $0.22 \pm 0.19$ ) exercise (threshold 0.5%). Bender, Unverzagt, Walker *et al* [1991] have reported comparable steady state peripheral blood CD34<sup>+</sup> levels of  $0.2 \pm 0.1\%$ , and felt sufficiently confident to study antigen expression within this population. The immunocytometry system was not considered sufficiently sensitive to permit valid study of CD34<sup>+</sup> HPC at this order of concentration.

### 3.2.3 Analysis of samples from normal bone marrow, mobilised peripheral blood and umbilical cord blood.

CD34<sup>+</sup> HPC comprised  $5.1 \pm 4.8\%$  of cells within the lymphoblastoid acquisition gate from bone marrow (n=8). Adult peripheral blood taken at day  $10 \pm 1$  post cyclophosphamide (PB<sup>m</sup>#1), showed a white count of  $1.9 \pm 0.6 \times 10^9/\text{l}$ , and contained  $6.8 \pm 4.3\%$  CD34<sup>+</sup> cells (n=7). Paired samples were taken at day  $12 \pm 1$  (PB<sup>m</sup>#2), showed a white count of  $16.7 \pm 3.1 \times 10^9/\text{l}$ , and contained  $5.3 \pm 5.3\%$  CD34<sup>+</sup> cells (n=8). Umbilical cord blood samples studied contained  $2.0 \pm 1.9\%$  CD34<sup>+</sup> cells (n=8), though this is not a representative group because cord blood samples with very low CD34<sup>+</sup> counts were discarded. The relative numbers of CD34<sup>+</sup> cells acquired were therefore  $510 \pm 480$  from BM,  $680 \pm 430$  from PB<sup>m</sup>#1,  $530 \pm 530$  from PB<sup>m</sup>#2, and  $200 \pm 190$  from CB. Clearly, less CD34<sup>+</sup> cells were analysed from cord blood samples, though this difference was not found to be statistically significant (Table 3.4). The contamination of the study population with non-specific labelled cells can be calculated from the ratio of the mean false %<sup>+</sup> in the negative control to the mean %CD34<sup>+</sup> ( $\times 100$ ). The respective contamination percentages were 9.5% (BM), 7.9% (PB<sup>m</sup>#1), 8.2% (PB<sup>m</sup>#2) and 20.6% (CB). Overall CD34%<sup>+</sup> cells are comparable with those reported by Bender for BM (1.8%) and PB<sup>m</sup> (0.6-5%), allowing for the effect of the lymphoblastoid gate [Bender, Unverzagt, Walker *et al.* 1991; Bender, Williams, Myers *et al.* 1992].

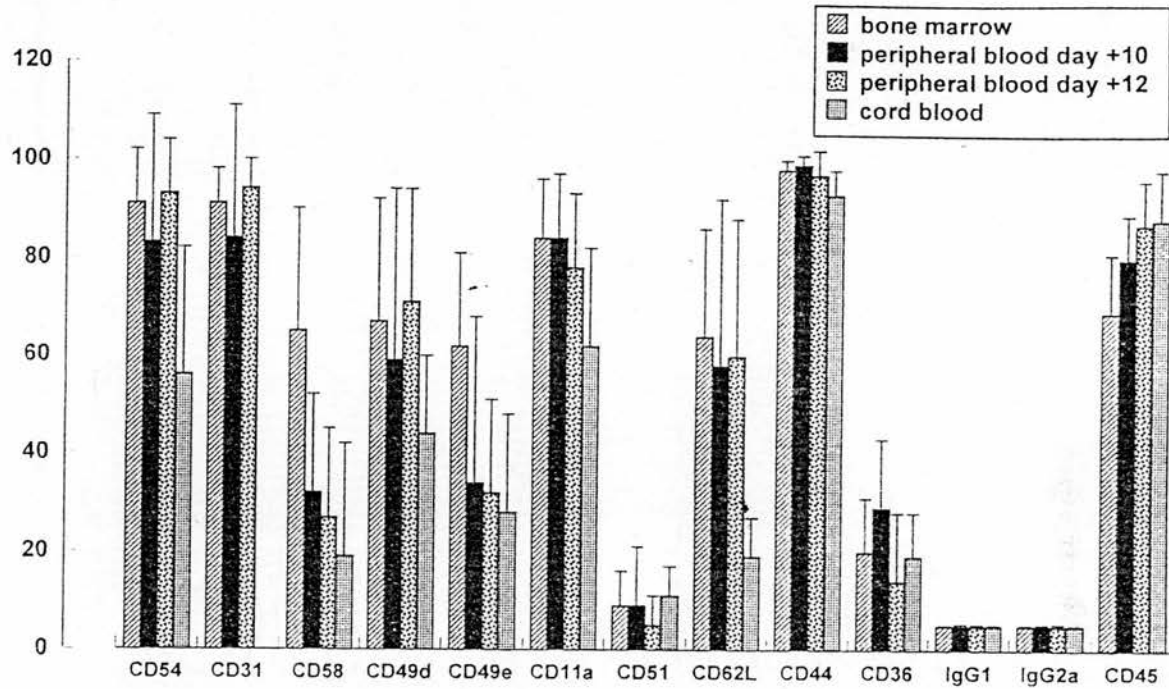
The results of CAM expression within these populations are summarised in Figures 3.1, 3.2 & 3.3 and Table 3.4. BM HPC expressed ICAM-1 (CD54), PECAM-1 (CD31), LFA-3 (CD58), VLA-4 (CD49d), VLA-5 (CD49e), LFA-1 (CD11a), L-Selectin (CD62L), HCAM (CD44) and CD36, but not VNR (CD51). Analysis of the mfi demonstrated very high HPC CD44 expression, with intermediate expression of the other CAMs with the exception of CD51. PB<sup>m</sup> #1 and #2 HPC display significantly less expression of CD58 and CD49e comparative to BM HPC, when analysed according to mean%fl<sup>+</sup> but no significant difference in the expression of other antigens. This difference was not detected by mfi, and may reflect reduced expression by, or reduced representation of a CD34<sup>+</sup> subpopulation. There was no significant difference in cell adhesion molecule expression by PB<sup>m</sup>1 and PB<sup>m</sup>2 HPC.

**Figure 3.1 Dual Immunocytometry Studies of Cell Adhesion Molecule Expression by Haematopoietic Progenitors.**



*Legend.* CD34 (FL-1) on the abscissa, CAMs (FL-2) on the ordinate. Quadrant 1: CD34<sup>+</sup> lin<sup>+</sup>; Q2: CD34<sup>+</sup> lin<sup>+</sup>; Q3: CD34<sup>+</sup> lin<sup>-</sup>; Q4: CD34<sup>+</sup> lin<sup>-</sup>. (A) ICAM-1 (CD54); (B) PECAM-1 (CD31); (C) LFA-3 (CD58); (D) VLA-4 (CD49d); (E) VLA-5 (CD49e); (F) LFA-1 (CD11a); (G) VNR (CD51); (H) L-Selectin (CD62L); (I) HCAM (CD44); (J) CD36; (K) CD45 positive control; (L) IgG negative control.

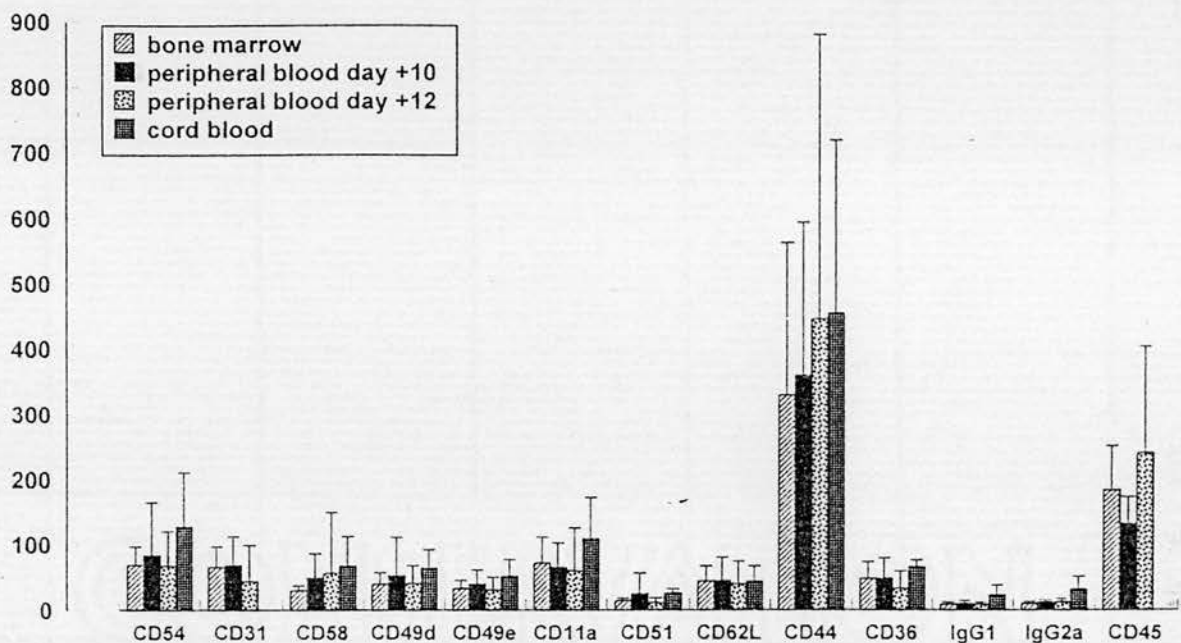
**Figure 3.2 Percentage Positivity Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood.**



*Legend.* Relative percentage positivity of cell adhesion molecule expression within the CD34<sup>+</sup> population from bone marrow, mobilised peripheral blood and cord blood. Results are reported as the mean  $\pm$  standard deviation of 7 or 8 replicate experiments. Detailed results are documented in Appendix 3 Table A4.



**Figure 3.3 Mean Fluorescence Intensity of Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood.**



*Legend.* Relative mean fluorescence intensity of cell adhesion molecule expression within the CD34<sup>+</sup> population from bone marrow, mobilised peripheral blood and cord blood. Results are reported as the mean  $\pm$  standard deviation of 7 or 8 replicate experiments. Detailed results are documented in Appendix 3 Table A5.

*Legend. Table 3.4 (overpage).* Bone marrow (BM) CD34<sup>+</sup> CAM expression is compared to that of mobilised peripheral blood at 2 time points (PB<sup>m</sup>#1 and PB<sup>m</sup>#2), and to that of cord blood (CB) by non-paired *t* test. PB<sup>m</sup>#1 and PB<sup>m</sup>#2 are compared by paired *t* test. Both percentage positivity and mean fluorescence intensity (mfi) in parentheses have been analysed. Differential expression of LFA-3 (CD58) and VLA-5 (CD49e) was the most consistent finding (see text for discussion).

**Table 3.4 Comparative Statistics on CD34<sup>+</sup> Cell Adhesion Molecule Expression.**

<i>CD</i>	<i>BM:PB<sup>m</sup>#1</i>	<i>BM:PB<sup>m</sup>#2</i>	<i>BM:CB</i>	<i>PB<sup>m</sup>#1:PB<sup>m</sup>#2</i>
CD34	ns	ns	ns	ns
CD54% (mfi)	ns (ns)	ns (ns)	p<0.01 (ns)	ns (ns)
CD31% (mfi)	ns (ns)	ns (ns)		ns (ns)
CD58% (mfi)	p<0.01 (ns)	p<0.01 (ns)	p<0.01 (P<0.05)	ns (ns)
CD49d% (mfi)	ns (ns)	ns (ns)	p<0.05 (ns)	ns (ns)
CD49e% (mfi)	p<0.05 (ns)	p<0.01 (ns)	p<0.01 (ns)	ns (ns)
CD11a% (mfi)	ns (ns)	ns (ns)	p<0.01 (ns)	ns (ns)
CD51% (mfi)	ns (ns)	ns (ns)	ns (p<0.01)	ns (ns)
CD62L% (mfi)	ns (ns)	ns (ns)	p<0.001 (ns)	ns (ns)
CD44% (mfi)	ns (ns)	ns (ns)	p<0.05 (ns)	ns (ns)
CD36% (mfi)	ns (ns)	ns (ns)	ns (ns)	p<0.05 (ns)
<i>FITC control</i>				
IgG1%	ns	ns	ns	ns
CD45%	ns	p<0.05	p<0.01	ns
<i>RPE control</i>				
IgG1% (mfi)	p<0.05 (ns)	ns (ns)	ns (p<0.05)	p<0.05 (ns)
IgG2a% (mfi)	ns (ns)	ns (ns)	ns (p<0.01)	ns (ns)
CD45%	ns	p<0.05	p<0.05	ns

CB HPC display significantly less expression of CD54, CD58, CD49e, CD11a and CD62L when analysed by %fl<sup>+</sup> but not by mfi. A central concern in the interpretation of this data was that the CB IgG1 and IgG2a SAM-RPE negative controls demonstrated significantly increased background binding compared to BM and PB samples (Figure 3.2 & 3.3, Table 3.4). In contrast the IgG1-FITC direct conjugate negative control showed little difference between these sources, suggesting that the major problem was overbinding of the second step reagent.

#### *3.2.4 Effects of mouse serum on binding of second-step reagent.*

A concern was raised that the use of mouse serum to blockade SAM- RPE uptake of QBEND10-FITC may reduce antibody binding, in particular of the SAM-RPE to the purified mAb. An experiment was carried out by dual labelling normal peripheral blood MNC with CD34-FITC and CD45, SAM-RPE. Omitting mouse serum from the protocol led to marked binding of the CD34 mAb by the second step reagent (Table 3.5). A triplicate experiment confirmed that mouse serum had no significant effect on CD45 SAM-RPE labelling of PB-MNC (Table 3.6).

#### *3.2.5 Comparability of data, and methodological problems.*

An important issue was considered to be the comparability of the data between different samples, linked to differences in fluorescence of the negative control, and variability in the size of the study population. A specific source of concern was that variation in autofluorescence or non-specific binding of antibodies, reflected by differences in the mfi of the negative controls, would lead to artefactual differences in % binding between sources due to shift in the discriminatory marker. Marker position (fluorescence channel) for each of the sources is documented in Table 3.7. Significance of differences between marker positions was calculated by non-paired *t* test. There was no significant difference between marker positions on FL-1, implying that CD34 definition is directly comparable. In FL-2, PB and BM markers were comparable, but the markers on CB were positioned significantly higher. This was not related to increased autofluorescence in this population, and reflected non-specific

**Table 3.5 Effects of Incubation with and without Mouse Serum.**

<i>Mouse serum</i>	CD34 <sup>+</sup> 0.46%	IgG1-FITC 0.46%
	CD45 <sup>+</sup> 94.87%	IgG1SAM-RPE 0.81%
<i>No serum</i>	CD34 <sup>+</sup> 55.64%	IgG1-FITC 1.43%
	CD45 <sup>+</sup> 88.20%	IgG1 SAM-RPE 1.08%

*Legend.* Data from a representative experiment demonstrating that omission of mouse serum from the labelling protocol leads to marked uptake of the CD34 mAb by the SAM-RPE second step reagent.

-----

**Table 3.6 Effect of Mouse Serum on CD45 SAM-RPE Labelling of Peripheral Blood Mononuclear Cells.**

<i>mouse serum</i>	CD45 <sup>+</sup> 56.2 ± 15.1% P<0.05	IgG1 SAM-RPE 3.6 ± 1.1%
<i>no serum</i>	CD45 <sup>+</sup> 71.8 ± 13.5% P<0.05	IgG1 SAM-RPE 6.3 ± 2.5%

*Legend.* Data presented is the mean ± standard deviation of 3 replicate experiments. Paired *t* test revealed no significant difference between CD45<sup>+</sup> or negative binding with or without mouse serum.

These two experiments demonstrated the general utility of mouse serum as a blocking agent in dual colour work.

**Table 3.7 Marker Channel for Each of the Sources.**

<i>fluorescence</i>	<i>BM (n=7)</i>	<i>PB<sup>m</sup>#1 (n=5)</i>	<i>PB<sup>m</sup>#2 (n=5)</i>	<i>CB (n=7)</i>
FL-1 auto	38 ± 10	25 ± 10 ns	18 ± 6 P<0.01	32 ± 25 ns
IgG1-FITC	59 ± 19	50 ± 9 ns	46 ± 17 ns	68 ± 17 ns
FL-2 auto	24 ± 6	15 ± 7 P<0.05	12 ± 3 P<0.01	25 ± 22 ns
IgG1 SAM-RPE	20 ± 7	21 ± 5 ns	21 ± 8 ns	53 ± 12 P<0.001
IgG2a SAM-RPE	22 ± 7	29 ± 7 ns	33 ± 14 ns	59 ± 23 P<0.01

*Legend.* Data presented is the mean channel position ± standard deviation for each source, autofluorescence and negative controls. The marker position used in PB<sup>m</sup>#1, PB<sup>m</sup>#2 and CB are compared to that used on BM by non-paired *t* test. There are few differences in the marker positions used on BM and PB<sup>m</sup>, but those used on CB samples were placed significantly higher due to higher non-specific uptake of the second step reagent.



binding of the purified mAb and / or second step reagent despite equivalent handling to that of other samples. Whether this is due to higher Fc receptor expression on the background population [Anderson & Looney, 1986], or the presence of unusual contaminants such as Wharton's Jelly in the cord blood samples is unclear [Flanagan & Mitoma, 1958]. Comparability of the CB data with BM and PB<sup>m</sup> data is therefore confounded by a smaller CD34<sup>+</sup> population, with greater false-positive contamination, and higher non-specific binding of the reagents in FL-2. The observations on BM and PB samples were therefore held to be valid, but the observations on CB were considered more difficult to interpret, especially in the presence of a generalised reduction in CAM expression, but significantly higher CD45 expression.

The biological significance of the observed differences between BM and PB<sup>m</sup> samples was considered to be uncertain for two reasons. First, differences in the nature of the source material including exposure to chemotherapy and supra-physiological levels of rhuG-CSF may engender differences in HPC heterogeneity between study groups. If HPC subsets express different levels of CAMs this could explain the observed differences in CAM expression between groups. Second, the functional role of the observed CAM expression in HPC-stromal and endothelial adhesion is poorly understood. This study was therefore instrumental in formulating both the objectives and the methodological design of further experiments.

### **3.3 Haematopoietic Progenitor Cell Expression of Lineage and Activation Markers.**

The pattern of activation and lineage-associated antigen expression within HPC has been discussed in Section 1.2.4. The panel of antigens used to discriminate HPC subsets is detailed in Table 3.8. The earliest identified lineage-associated antigens were used as markers for lineage commitment along with HLA-DR and CD38 as markers of HPC activation [Fraser, Leahy & Berridge, 1986; Loken, Shah, Dattilio *et al.* 1987a & b; Civin & Loken, 1987; Terstappen, Hollander, Meiners *et al.* 1990;



**Table 3.8 Lineage and Activation Markers Studied.**

<i>CD</i>	<i>Lineage</i>	<i>mAb, isotype (species)</i>	<i>Source</i>
CD33	myeloid	D3HL60.251 IgG1 (murine)	Immunotech
CD7	T lymphoid	8H8 IgG1 (murine)	Immunotech
CD19	B lymphoid	J4.119 IgG1 (murine)	Immunotech
CD71	erythroid	YDJ1.2.2 IgG1 (murine)	Immunotech
CD41a	megakaryocytic	P2 IgG1(murine)	Immunotech
CD38	activation	T16 IgG1 (murine)	Immunotech
HLA-DR	activation	B8.12.2 IgG2b (murine)	Immunotech

---

Terstappen & Loken 1990; Terstappen, Safford & Loken, 1990; Srour, Brandt, Briddell *et al.* 1991; Terstappen, Huang, Safford *et al.* 1991; Terstappen, Huang & Pickler, 1992]. The limitations of this selection are discussed in Section 3.6.2.

### *3.3.1 Modifications in dual immunocytometry methodology.*

The methodological problems associated with the cell adhesion molecule study (Section 3.2.5) stimulated a number of modifications to the dual immunocytometry protocol aimed at reduction in procedure-related cell loss or ignorance, reduction in non-specific binding of labelling reagents, more stringent definition of a larger study population and more informative analysis of that population.

The immunocytometry protocol (Table 3.2) was modified in the following ways:

- (1) Contemporaneous whole blood and leucapheresis products were examined from the same patient, in case the leucapheresis procedure had selectively depleted a CD34<sup>+</sup> subpopulation.
- (2) A whole-sample labelling technique was used with red cell lysis, in order to reduce Ficoll-related cell losses (Chapters 2.4.4 and 6.6.3).
- (3) 0.5% human gamma globulin solution was used instead of rabbit serum, to improve Fc receptor blockade.
- (4) The number of washes was reduced, but the volume of each wash increased (to reduce preparation time and cell trauma).
- (5) 8G12-RPE was used instead of QBEND-FITC as the anti-CD34 label because of

its superior resolution index, allowing a more stringent marker to be set (0.05%), with consequent reduction in contamination of the study population (Chapter 2.4.6 and 2.9.3). (6) Directly conjugated mAbs were used as lineage markers, to reduce non-specific binding. (7) All mAb incubation steps were reduced to 15min at +4°C to improve the ratio of specific to non-specific binding. (8) The Lysis II software was used rather than Consort 30 software, because of added flexibility. (9) The lymphoblastoid acquisition gate was established by backgating the CD34<sup>+</sup> population onto the SSC / FSC dot plot, to allow more accurate placement (Figure 3.4). (10) 30,000 cells were acquired per analysis to allow a larger study population (the low frequency of a cell population leads to a high cv which may skew the mean [Terstappen, Buescher, Nguyen *et al.* 1993]). (11) Additional negative and positive controls were established (*i.e.* CD34-RPE, IgG1-FITC and CD34-RPE, CD45-FITC) in order to allow more accurate determination of mfi within the CD34<sup>+</sup> population. (12) A marker was established at the point of minimal inflexion on the FITC histogram, to establish greater flexibility and sensitivity in detecting lineage marker expression.

The modified protocol and test system are summarised in Table 3.9 and 3.10.

### 3.3.2 Results.

The results of this series of experiments is presented in Figures 3.5, 3.6 & 3.7 and Table 3.11. Improvements in assay technique led to improvements in the number of CD34<sup>+</sup> cells examined, and a reduction in the false-positive contamination of the study population. Whole blood samples (PB<sup>m</sup> and CB) were not as easy to study as BM or LP because the red cell burden was greater and interfered with both the staining and the analytical technique. The modifications in technique allowed better standardisation of marker position, but CB samples still demonstrated a significantly smaller CD34<sup>+</sup> study population and significantly greater contamination compared to other sources. Under global analysis no significant expression of CD33, CD7, CD19 or CD41a by CD34<sup>+</sup> HPC from any source was detected (Appendix 3 Tables A6 & A7). However, direct inspection of

**Table 3.9 Modified Dual Immunofluorescence Labelling Protocol used for the Analysis of Lineage-Marker Expression.**

*Immunofluorescence labelling.*

whole product (in anticoagulant medium)

$5 \times 10^5$  nucleated cells / test.

wash x2 in 1ml handling medium

incubate with 0.5ml of 0.5% human gamma globulin for 15min

incubate with 10 $\mu$ l 8G12-RPE + 10 $\mu$ l mAb-FITC for 15min at +4 $^{\circ}$ c

wash x1 in 1ml of handling medium

incubate in 2ml of erythrocyte lysis solution for 15min

resuspend in 0.5ml 1% paraformaldehyde

*Flow cytometry.*

Standard cytometer set up used (Table 2.6).

A test sample of cells was acquired ungated in SetUp. The CD34 $^{+}$  population was defined on the FL2 histogram, and highlighted on the FSC / SSC dot-plot. A lymphoblastoid acquisition gate was established on the FSC / SSC dot-plot using the CD34 $^{+}$  population as a whole.

**Table 3.10 Test System for the Lineage Marker Study.**

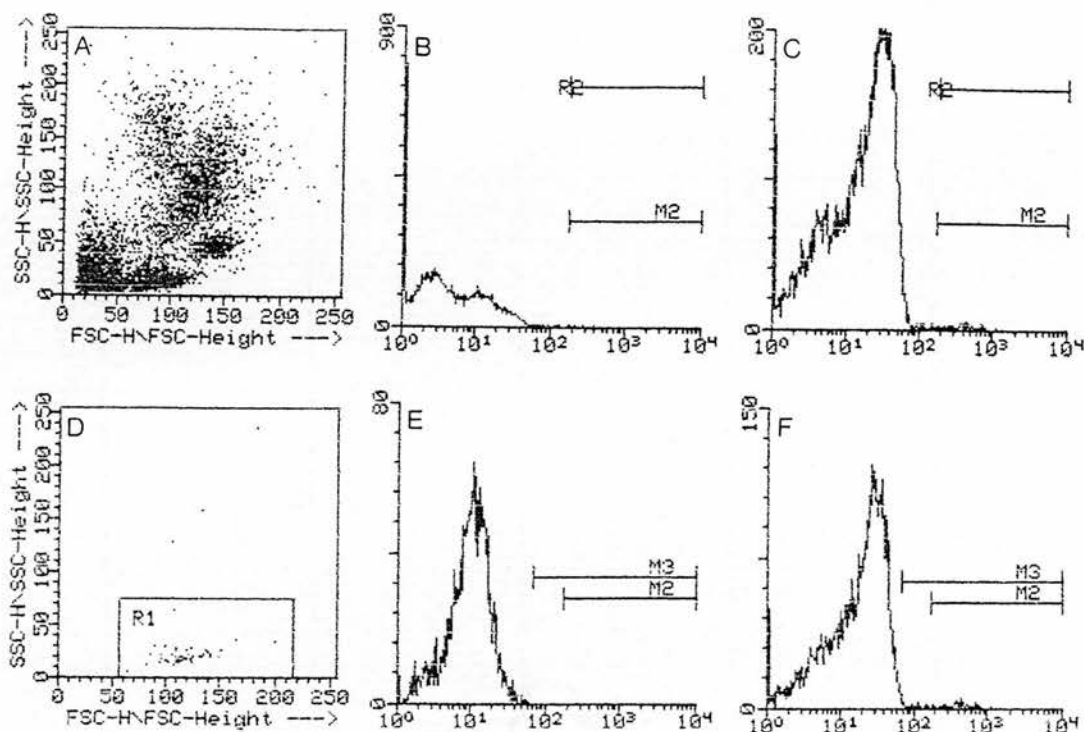
1. CD33-FITC	8G12-RPE
2. CD7-FITC	8G12-RPE
3. CD19-FITC	8G12-RPE
4. CD71-FITC	8G12-RPE
5. CD41a-FITC	8G12-RPE
6. CD38-FITC	8G12-RPE
7. HLA-DR-FITC	8G12-RPE
8. CD45-FITC	
9.	CD45 SAM-RPE
10. Autofluorescent.	
11. IgG1-FITC	
12.	IgG1-RPE
13. IgG1-FITC	8G12-RPE
14. CD45-FITC	8G12-RPE

*Legend.* 8G12-RPE: anti-CD34 mAb directly conjugated with RPE.

CD\*\*-FITC: mAb to a lineage or activation marker directly conjugated to FITC.

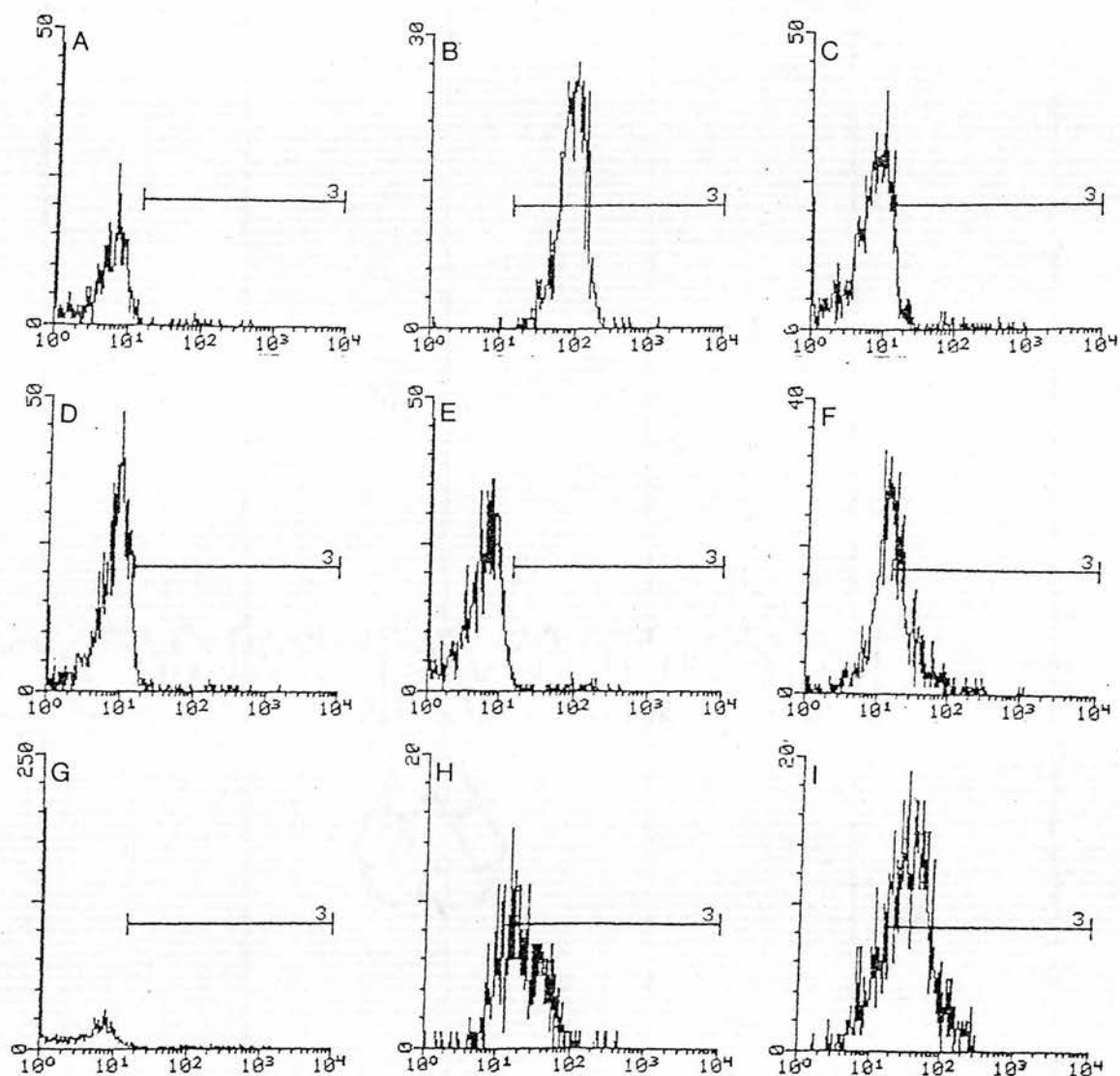
Labelled samples were compared with similarly conjugated mAbs of the same isotype but irrelevant specificity.

**Figure 3.4 Backgating of the CD34<sup>+</sup> Population on the FSC / SSC Dot-Plot.**



*Legend.* Lysed peripheral blood leukocytes acquired on an open gate (A) demonstrated negative control fluorescence (M2) of 0.05% (B), CD34<sup>+</sup> 1.67% (C). A gate (R2) established on the CD34<sup>+</sup> population allows these cells to be projected on the FSC / SSC dot-plot and an acquisition gate (R1) to be drawn around the lymphoblastoid population (D). Negative control fluorescence (M3) on the R1 population is now 0.00% (E) and CD34<sup>+</sup> 3.25% (F).

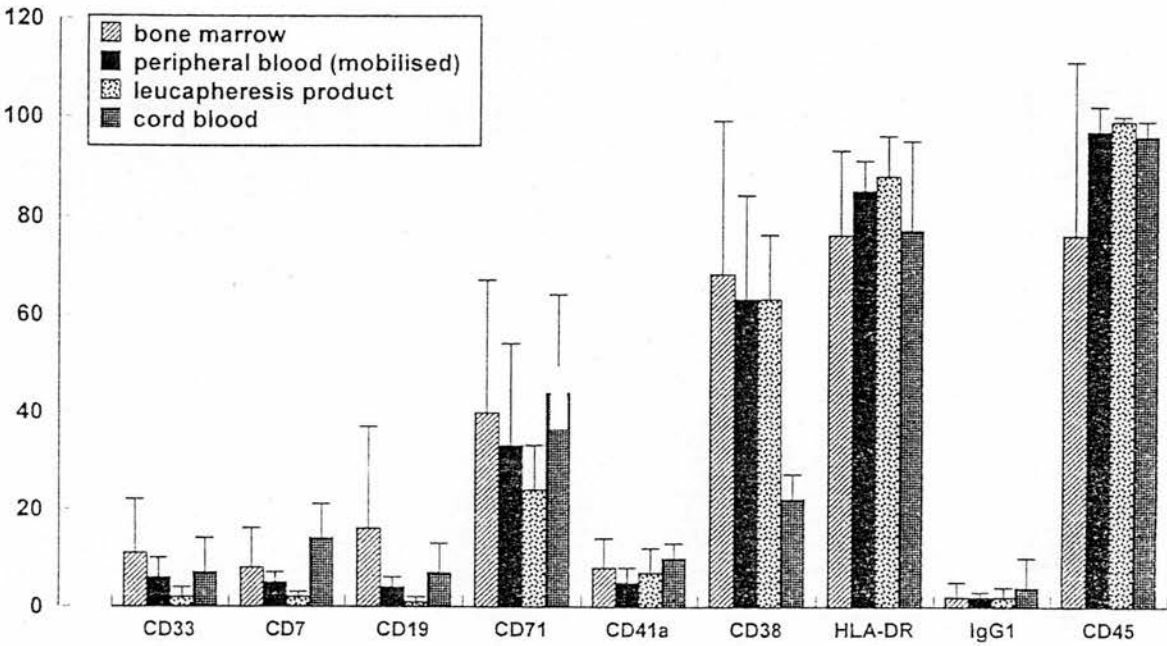
Figure 3.5 Representative Experiment Demonstrating Lineage Marker Expression by Haematopoietic Progenitors.



*Legend.* Cells were acquired and the CD34<sup>+</sup> population identified as described in Figure 3.4. Cells gated on the CD34<sup>+</sup> population (M2 Figure 3.4F) were projected onto the FL-1 histogram. (A) negative control %fl<sup>+</sup> 3.1% mfi 10. (B) CD45 %fl<sup>+</sup> 99.6% mfi 95. (C) CD33 %fl<sup>+</sup> 10.3% mfi 14. (D) CD7 %fl<sup>+</sup> 6.8% mfi 14. (E) CD19 %fl<sup>+</sup> 4.2% mfi 10. (F) CD71 %fl<sup>+</sup> 50.0% mfi 26. (G) CD41a %fl<sup>+</sup> 5.8% mfi 15. (H) CD38 %fl<sup>+</sup> 66.2% mfi 30. (I) HLA-DR %fl<sup>+</sup> 82.4% mfi 44.

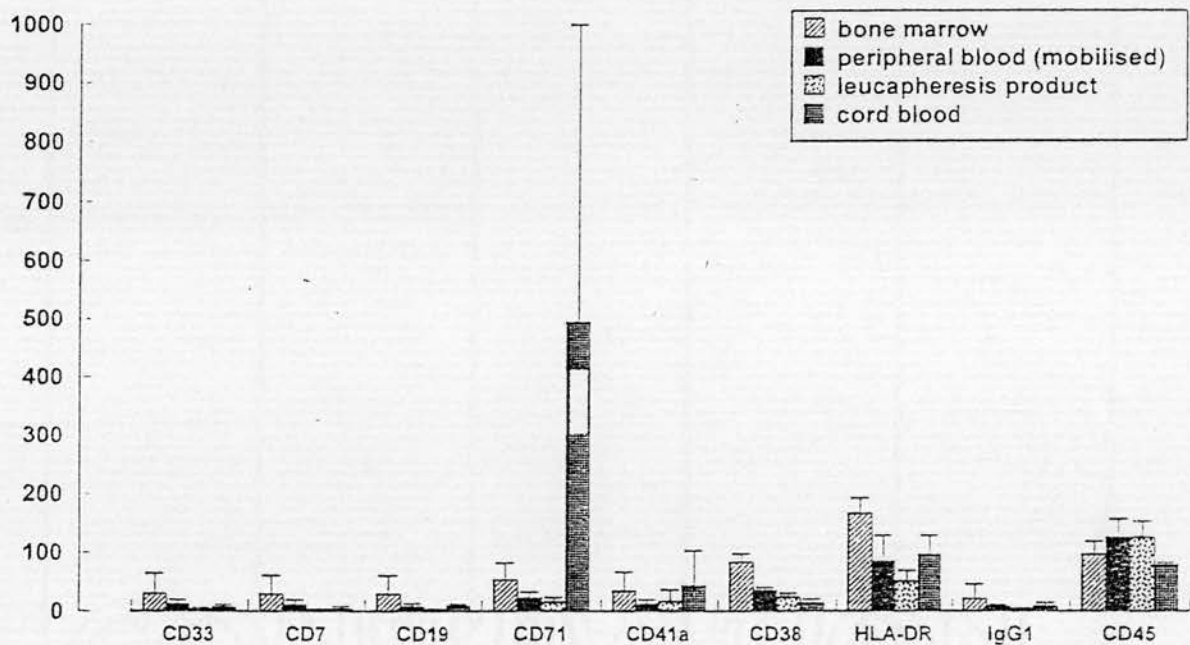


**Figure 3.6 Percentage Positive Lineage Marker Expression within the CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood.**



*Legend.* Relative percentage positivity of lineage and activation marker expression within the CD34<sup>+</sup> population from bone marrow, mobilised peripheral blood and cord blood. Results are reported as the mean  $\pm$  standard deviation of 4 replicate experiments. Detailed results are documented in Appendix 3 Table A6.

Figure 3.7 Mean Fluorescence Intensity of Lineage Marker Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Cord Blood.



*Legend.* Relative percentage positivity of lineage and activation marker expression within the CD34<sup>+</sup> population from bone marrow, mobilised peripheral blood and cord blood. Results are reported as the mean  $\pm$  standard deviation of 4 replicate experiments. Detailed results are documented in Appendix 3 Table A7.

*Legend. Table 3.11 (overpage).* Bone marrow (BM) CD34<sup>+</sup> lineage and activation marker expression is compared to that of mobilised peripheral blood (PB<sup>m</sup>), leucapheresis product (LP) and cord blood (CB) by non-paired *t* test. PB<sup>m</sup> and LP are compared by paired *t* test. Both percentage positivity and mean fluorescence intensity (mfi) in parentheses, have been analysed. See text for discussion.

**Table 3.11 Comparative Statistics on Lineage Marker Expression by CD34<sup>+</sup> Cells from Bone Marrow, Mobilised Peripheral Blood and Matched Leucapheresis Product and Cord Blood.**

<i>CD</i>	<i>BM:PB<sup>m</sup></i>	<i>BM:LP</i>	<i>PB<sup>m</sup>:LP*</i>	<i>BM:CB</i>
CD34	ns	ns	p<0.05	p<0.01
Events	p<0.05	ns	p<0.05	p<0.01
IgG1-RPE	p<0.05	ns	ns	ns
Contamination.	ns	ns	ns	p<0.05
CD33% (mfi)	ns (ns)	ns (ns)	ns (ns)	ns (ns)
CD7% (mfi)	ns (ns)	ns (ns)	ns (ns)	ns (ns)
CD19% (mfi)	ns (ns)	ns (ns)	p<0.05 (ns)	ns (ns)
CD71% (mfi)	ns (ns)	ns (P<0.05)	ns (ns)	ns (ns)
CD41a% (mfi)	ns (ns)	ns (ns)	ns (ns)	ns (ns)
CD38% (mfi)	ns (p<0.001)	ns (p<0.001)	ns (p<0.05)	p<0.05 (p<0.001)
HLADR (mfi)	ns (p<0.02)	ns (p<0.001)	ns (ns)	ns (p<0.05)
IgG1% (mfi)	ns (ns)	ns (ns)	ns (ns)	ns (ns)
CD45% (mfi)	ns (ns)	ns (ns)	ns (ns)	ns (ns)

the relevant fluorescence histograms (Figures 3.5 - 3.7) suggest that CD33 and CD19 are expressed by subpopulations of CD34<sup>+</sup> cells within BM from at least some individuals, but not by circulating populations. This information is masked by the high variance between samples and the relatively low power of the study (n=4). CD71 is expressed by 40% of BM HPC and somewhat fewer circulating cells. CD38 and HLA-DR are expressed by the majority of HPC, but at higher antigen density in BM compared to PB populations. There is a particularly marker difference in the expression of CD38 between CB and other populations. There are therefore detectable differences in the expression of lineage and activation markers between normal bone marrow and circulating HPC (Table 3.11).

### **3.4 Characterisation of Cell Adhesion Molecule, Lineage and Activation Marker Expression by Human Haematopoietic Cell Lines.**

In view of the difficulties inherent in studying CAM expression within HPC subsets (*vide infra*), it was decided to study a panel of human haematopoietic cell lines to ascertain whether differences in lineage or activation markers were associated with differences in CAM expression. The cell lines KG1a, K562, HL-60, NALM-6 and CEM were used for this study. Their characteristics are listed in Table 2.1 and culture and storage discussed in Section 2.2.2.

Single colour immunofluorescence was used to characterise the pattern of expression of CAMs using purified mAbs and a SAM-FITC second step reagent (Section 3.2), and of lineage / activation markers using directly FITC conjugated mAbs (Section 3.3). Standard immunocytometry labelling technique was used with the omission of erythrocyte lysis (Chapter 2.4 and Table 2.10). The settings on the flow cytometer were adjusted to compensate for the larger size and greater autofluorescence of the cell lines compared to normal human cells (Table 3.12). 10,000 events were acquired through an acquisition gate set on the FSC / SSC dot-plot to include the singlet population, but to exclude cellular aggregates and debris (Figure 2.6).

The results of this work are summarised in Figures 3.8 to 3.12 inclusive. KG1a is a human acute myelogenous leukaemia cell line. The cells are CD34<sup>+</sup> and coexpress CD7 and CD71, though not CD38, HLA-DR or CD33. In addition they express the CAMs CD54, CD31, CD58, CD49d, CD49e, CD11a, CD44 and CD36. K562 is a human chronic myelogenous leukaemia cell line. The cells are CD34<sup>-</sup>, CD19<sup>+</sup> and CD71<sup>+</sup>. They co-express CD54, CD58, CD49d, CD49e and CD51. HL-60 is a human promyelocytic leukaemia cell line. They are CD34<sup>-</sup>, HLA-DR<sup>+</sup>, and co-express CD54, CD58, CD49d, CD49e, CD11a and CD44. NALM-6 is a human B lymphoid cell line, which is CD34<sup>-</sup>, CD19<sup>+</sup>, CD71<sup>+</sup>, CD38<sup>+</sup> and HLA-DR<sup>+</sup>. Interestingly, this line is CD45<sup>-</sup>. The cells coexpress CD54, CD58, CD49d and CD49e. Finally, CEM is a human acute T lymphoblastic leukaemia cell line, CD34<sup>-</sup>, CD7<sup>+</sup>, CD71<sup>+</sup> and CD38<sup>+</sup>. The cell coexpress CD54, CD49d, CD49e and CD44. L-Selectin (CD62L) is thought to be shed from activated leucocytes by proteolytic cleavage, and perhaps it is not suprising, therefore, that it was not found to be expressed by any of the cell lines.

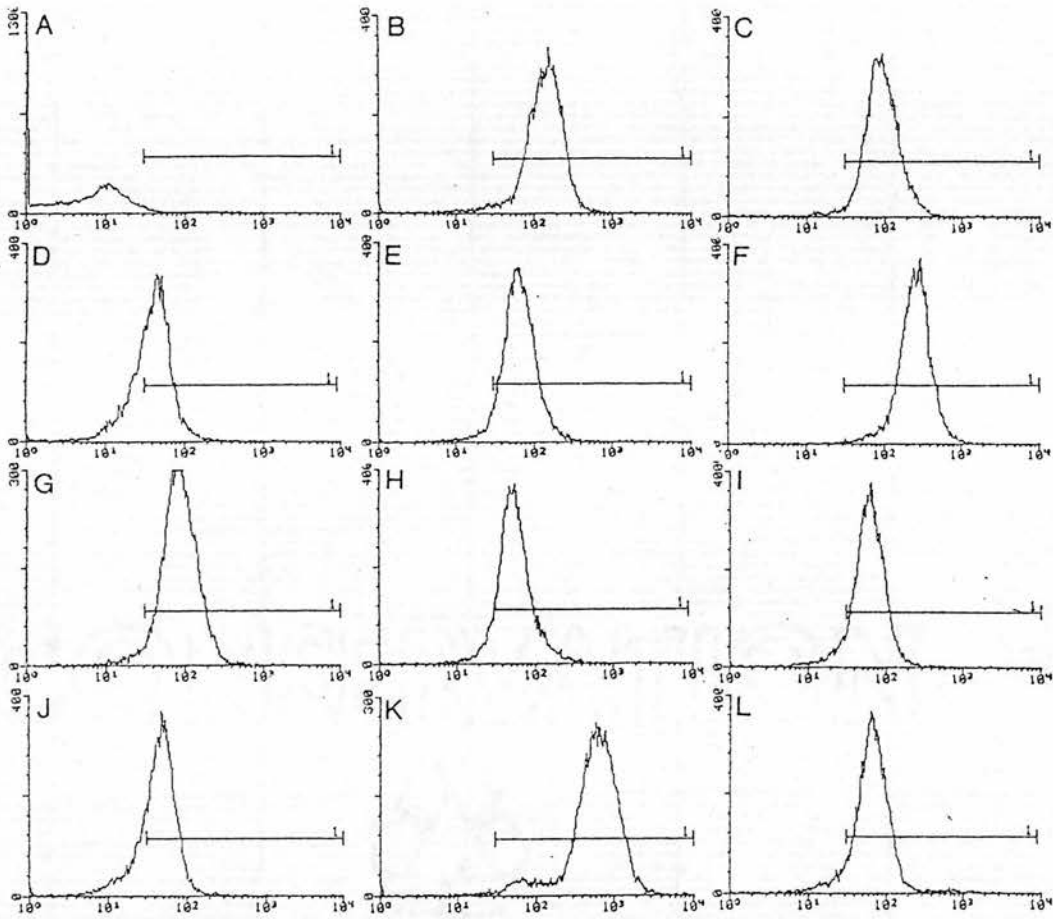
This study supported the generalisation that differences in CAM expression do occur in association with differences in activation and lineage commitment. For example, the composite data shows that CD54, CD49d and 49e were expressed by all cell lines, whereas CD11a and CD44 were not expressed by CD19<sup>+</sup> cell lines (K562 and NALM-6), and CD31 and CD36 expression was restricted to the CD34<sup>+</sup> cell line KG1a. Unfortunately, it is difficult to know the extent to which cell lines can be considered representative of HPC subsets [Akiyama, Larjava, Yamada *et al.* 1990].

**Table 3.12 Flow Cytometry Settings for the Cell Line Studies.**

<i>Parameter</i>	<i>Detector</i>	<i>Amplification</i>	<i>Threshold</i>
FSC	E00	1.00	52
SSC	340	1.00	
FL-1	475	Log	
FL-2	475	Log	
<i>Compensation</i>			
FL1 - %FL2	1.8%		
FL2 - %FL1	42.0%		

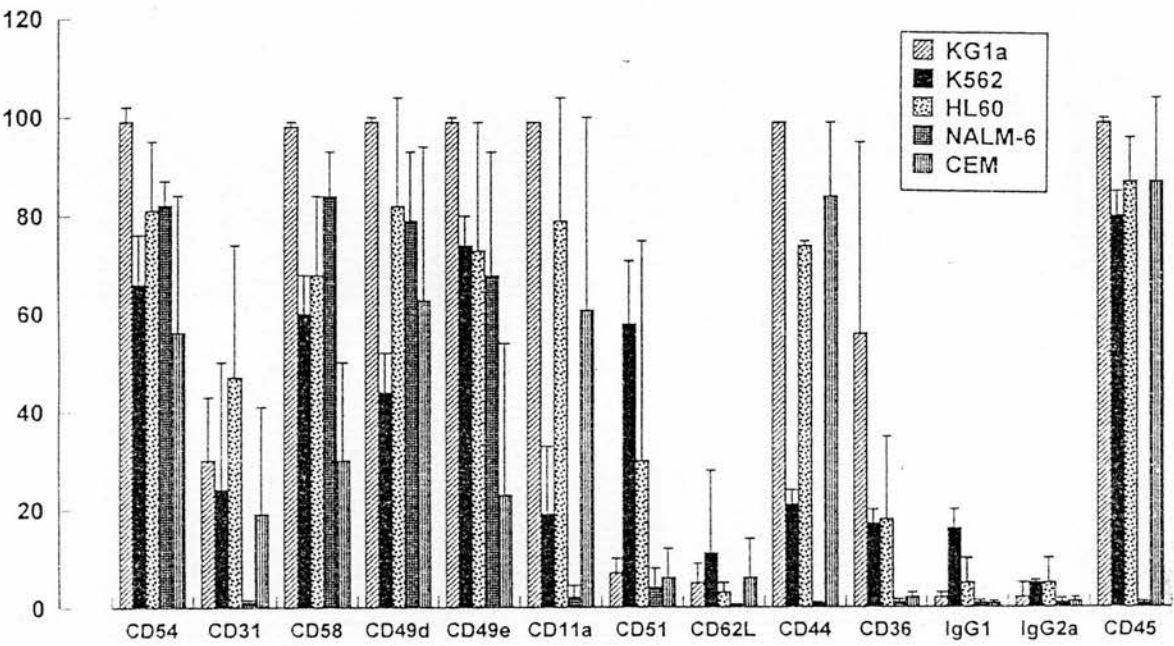


**Figure 3.8 Representative Experiment Demonstrating Single Colour Immunofluorescence of Cell Adhesion Molecule Expression by KG1a.**



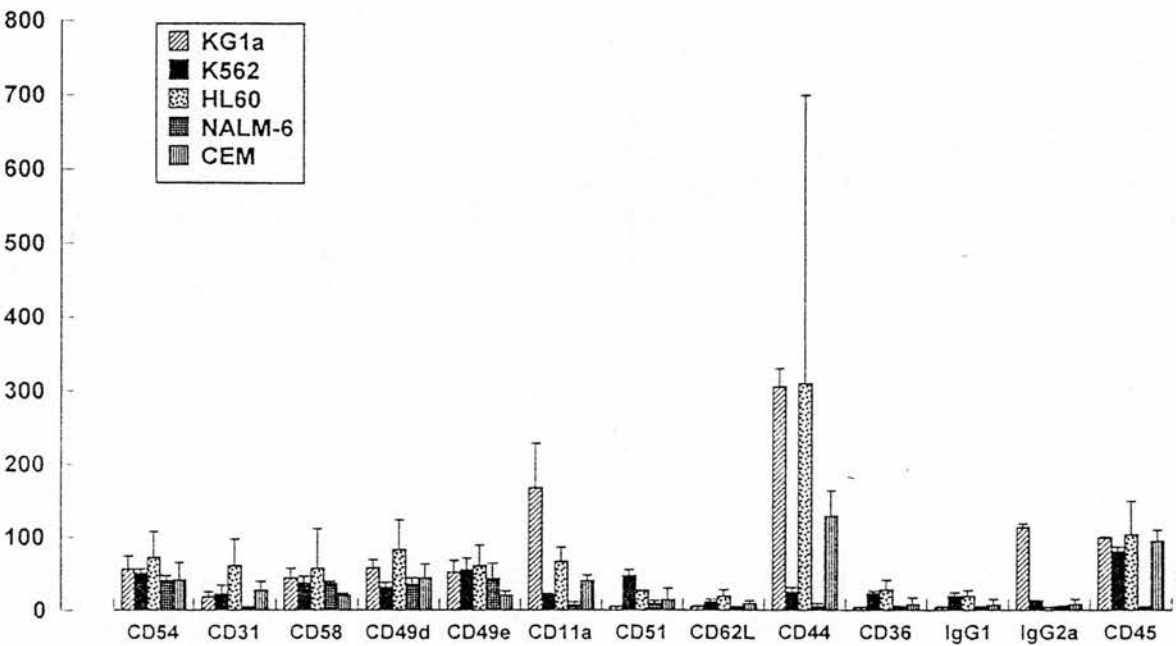
*Legend.* KG1a labelled with purified mAbs to CAMs and sheep anti-mouse FITC. (A) negative control %fl<sup>+</sup> 4.5% mfi 12. (B) CD45 %fl<sup>+</sup> 96.6% mfi 150. (C) ICAM-1 (CD54) %fl<sup>+</sup> 97.4% mfi 104. (D) PECAM-1 (CD31) %fl<sup>+</sup> 69.8% mfi 44. (E) LFA-3 %fl<sup>+</sup> 94.7% mfi 73. (F) VLA-4 (CD49d) %fl<sup>+</sup> 96.3% mfi 95. (G) VLA-5 (CD49e) %fl<sup>+</sup> 91.0% mfi 64. (H) LFA-1 (CD11a) %fl<sup>+</sup> 99.6% mfi 274. (I) VNR (CD51) %fl<sup>+</sup> 91.4% mfi 66. (J) L-Selectin (CD62L) %fl<sup>+</sup> 77.3% mfi 48. (K) HCAM (CD44) %fl<sup>+</sup> 98.5% mfi 679. (L) CD36 %fl<sup>+</sup> 92.5% mfi 80.

**Figure 3.9 Percentage Positive Cell Adhesion Molecule Expression by Haematopoietic Cell Lines.**



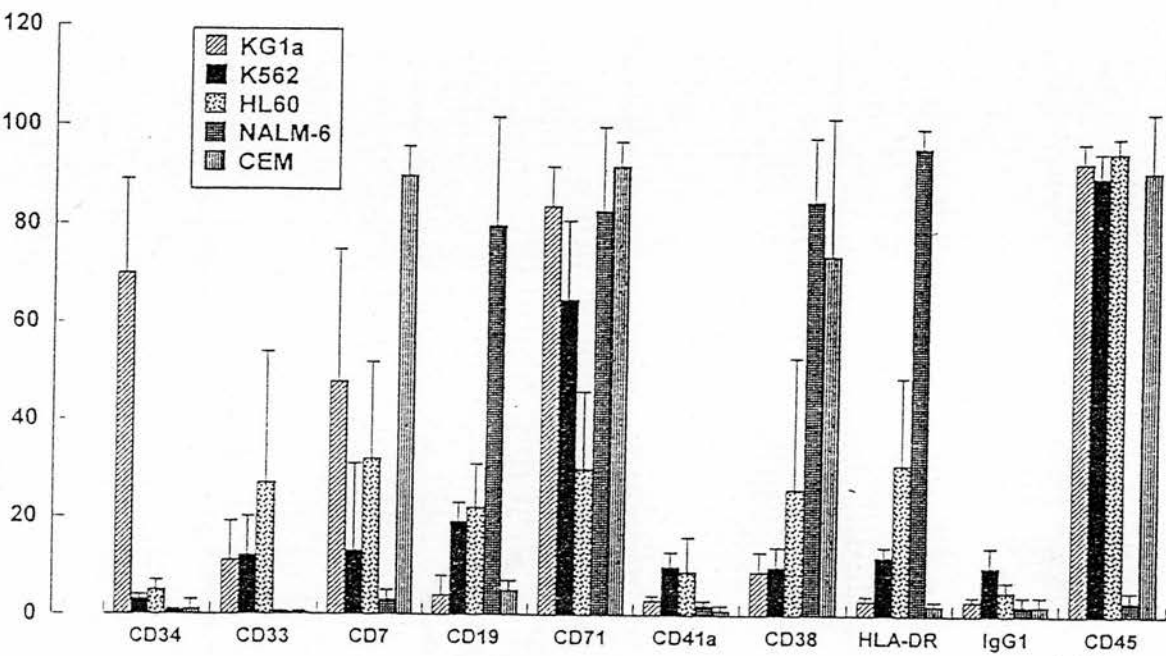
*Legend.* Relative percentage positivity of cell adhesion molecule expression by KG1a, K562, HL-60, NALM-6 and CEM. Results are reported as the mean  $\pm$  standard deviation of 4 replicate experiments. Detailed results are documented in Appendix 3 Table A8-12.

**Figure 3.10 Mean Fluorescence Intensity of Cell Adhesion Molecule Expression by Haematopoietic Cell Lines.**



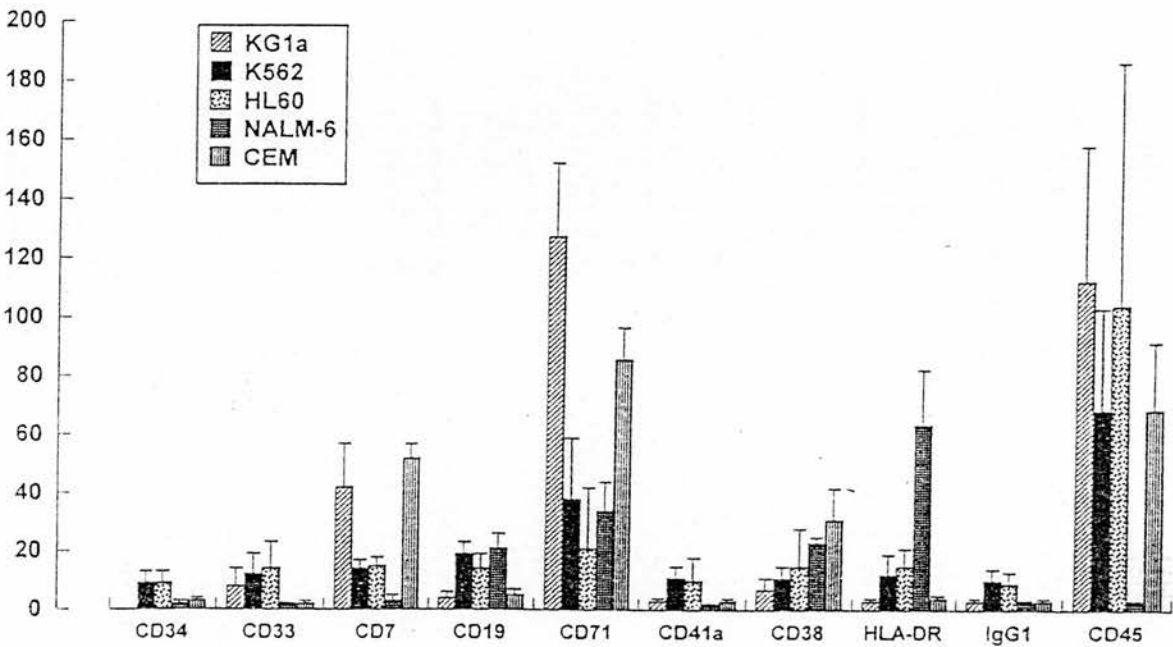
*Legend.* Relative mean fluorescence intensity of cell adhesion molecule expression by KG1a, K562, HL-60, NALM-6 and CEM. Results are reported as the mean  $\pm$  standard deviation of 4 replicate experiments. Detailed results are documented in Appendix 3 Table A8-12.

**Figure 3.11 Percentage Positive Lineage and Activation Marker Expression by Haematopoietic Cell Lines.**



*Legend.* Relative percentage positivity of lineage and activation marker expression by KG1a, K562, HL-60, NALM-6 and CEM. Results are reported as the mean  $\pm$  standard deviation of 4 replicate experiments. Detailed results are documented in Appendix 3 Table A8-12.

**Figure 3.12 Mean Fluorescence Intensity of Lineage and Activation Marker Expression by Haematopoietic Cell Lines.**



*Legend.* Relative mean fluorescence intensity of lineage marker expression by KG1a, K562, HL-60, NALM-6 and CEM. Results are reported as the mean  $\pm$  standard deviation of 4 replicate experiments. Detailed results are documented in Appendix 3 Table A8-12.

### **3.5 Development of Three Colour Immunocytometry to Study Cell Adhesion Molecule Expression within Haematopoietic Progenitor Subsets.**

It was clear, therefore, that the pattern of CAM expression within CD34<sup>+</sup> lineage subsets would have to be defined using three-colour immunocytometry. This provided a particularly difficult technical and analytical challenge.

The immunofluorescence labelling protocol was fairly standard. 8G12-RPE was used to label the CD34<sup>+</sup> population on the grounds that the antigen present in the lowest density generally should be stained with the brightest fluorochrome. Directly FITC conjugated mAbs addressed to the lineage-associated antigens were used. Commercial availability dictated the use of anti-CAM mAbs in purified form, with an anti-murine third colour reagent. Binding of the directly conjugated reagents by the second-step (third colour) reagent was blocked with mouse serum as previously. The labelling protocol is summarised in Table 3.13. Up to 10,000 events were acquired through a gate defined on the CD34<sup>+</sup> / FSC dot plot (Lysis II software). This was necessary in order to substantially increase the number of cells under analysis, to include cells of high side scatter in the analysis, and in order to be able to carry out the nested analysis of CAM expression within lineage subsets which was not otherwise possible on the Lysis II software. The Paint-A-Gate software can carry out a nested analysis, but requires transfer of markers between programmes which is unsatisfactory, and provides inadequate statistical information.

Several technical problems required solution, the choice of third colour reagent and establishment of a satisfactory compensation network, the use of beads to calibrate the intensity of antigen expression, a more detailed estimation of the level of contamination of the study (CD34<sup>+</sup>) population, and an evaluation of the distribution of CD34<sup>+</sup> cells on the FSC / SSC dot plot.



**Table 3.13 Three Colour Immunofluorescence Labelling Protocol.**

1x10<sup>6</sup> leucocytes (whole blood)

washed twice in 1ml handling medium

incubated for 15min in 0.5% human immunoglobulin in PBS

incubated for 15min with 20µl anti-CAM mAb (purified)

washed twice in 1ml handling medium

incubated for 15min with 20µl of 2nd stage reagent

washed twice in 1ml handling medium

incubated for 15min with 20µl of 3rd stage reagent (Quantum Red only)

washed twice in handling medium (Quantum Red only)

incubated for 15min with 20µl murine serum

incubated for 15min with 20µl 8G12-RPE and 20µl Lin-FITC

washed once in 1ml handling medium

incubated for 5-10min with 0.5ml erythrocyte lysis solution (until clarification).

washed once in handling medium

stored in 1ml 1% paraformaldehyde.

### *3.5.1 Evaluation of potential third colour reagents.*

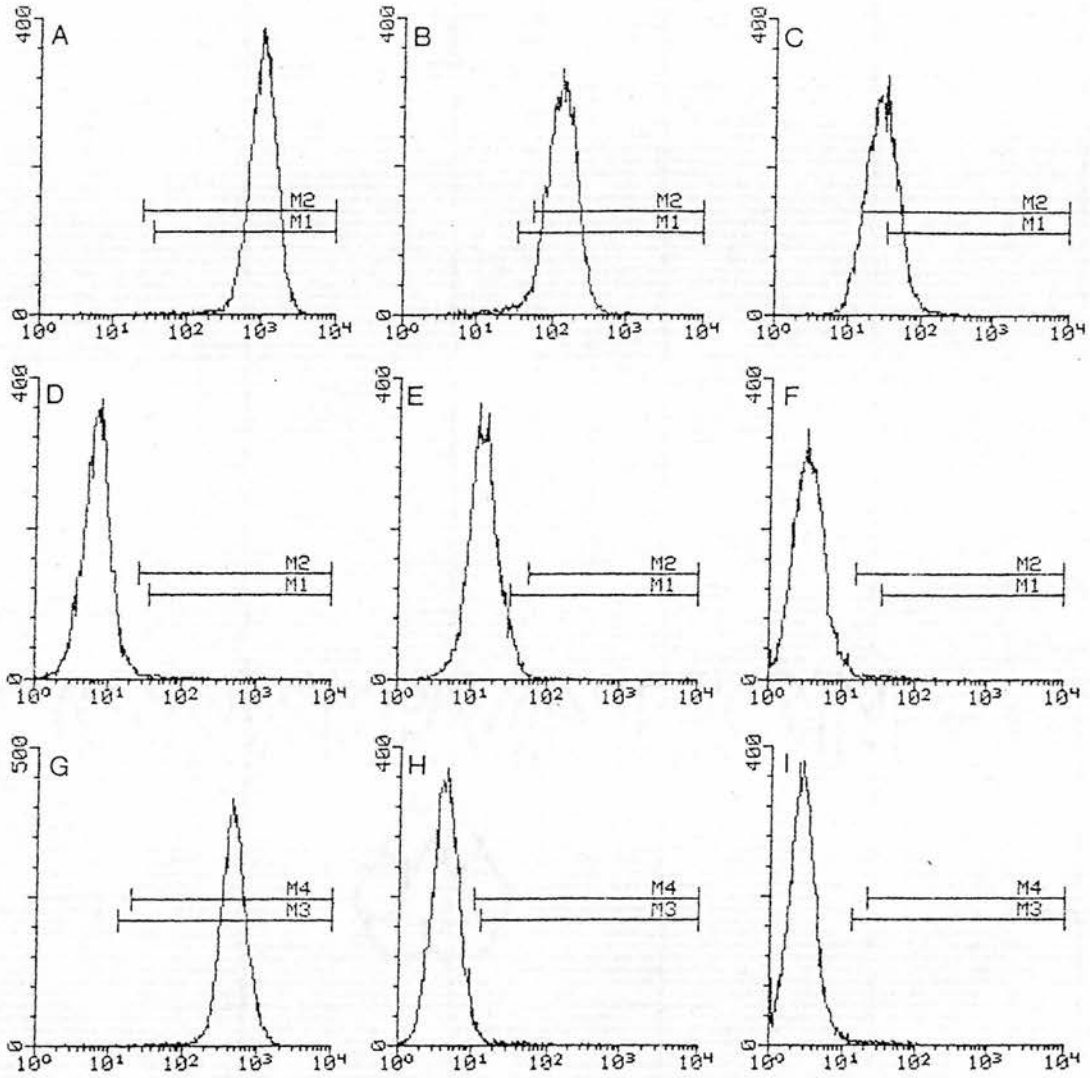
Three reagents were assessed for their utility as 3<sup>rd</sup> colour reagents: GAM-Red 613, streptavidin-Quantum Red, and RAM-PerCP. Whereas GAM-Red 613 and RAM-PerCP could be used by a classical two-step indirect labelling technique, a three step labelling procedure had to be adopted with Quantum Red whereby the purified mAb was labelled with a RAM-biotinylated second step, and the streptavidin-Quantum Red was used as a third step reagent. The 8G12-RPE and lineage-FITC mAbs were then added as a fourth step. Both the second and third step reagents had to be independently titrated.

The three reagents were evaluated by staining KG1a with the anti-CD44 mAb and with the appropriate second (and third) step reagent. CD44 was chosen as it was the most densely expressed of the CAMs (and therefore gave rise to the brightest fluorescence likely to be confronted), and because it was an IgG2a isotype mAb - like the LFA-3 and VLA-5 mAbs which were the ultimate target of the study. Negative controls were established with purified IgG2a of irrelevant specificity and the appropriate reagents as detailed above. Red 613 proved very bright (Figure 3.13, A & D), and engendered a high degree of fluorescence in FL-2 despite maximum compensation (G). In experiments on LP the shift in the FL-2 negative control completely obscured the RPE-labelled CD34<sup>+</sup> population. Both Quantum Red and PerCP provided acceptable fluorescence overlap (H & I), the FL-2 histograms differed little from those of the negative controls (not shown). Although the mfi of the Quantum Red negative control (E) was higher than that of PerCP (F), the combination of a higher mfi on the positive population (B *cf*: C) and a cleaner discrimination (probably due to the specificity of the streptavidin-biotin system), meant that the percentage positivity was greater with Quantum Red.

### *3.5.2 Use of calibration beads to assess the intensity of antigen expression.*

Quantum simply cellular calibration microbeads were used to provide calibration of antigen density. The beads are a mixture of five populations of microbeads, four with defined antibody binding capacity and one non-binding. The

**Figure 3.13 Spectral Overlap of the Third Colour Reagents.**

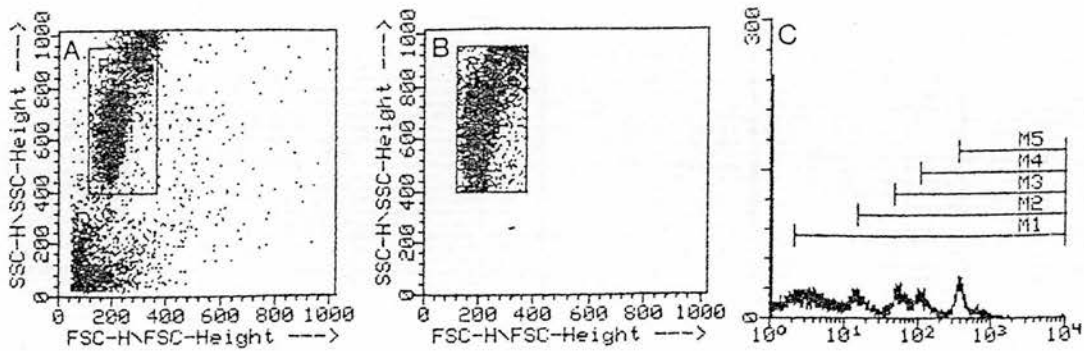


*Legend.* Sample of KG1a was labelled with CD44 and Red 613 (A, D & G), Quantum Red (B, E & H) or PerCp (C, F & I) as described in the text. A, B & C represent the positively stained samples (FL-3) and D, E & F their respective negative controls. M1 is a fixed marker, and M2 is set on 0.5% of the negative controls. G, H & I are the FL-2 histograms of the respective FL-3 labelled samples (A, B & C). Once again M3 is a fixed marker and M4 is set on 0.5% of the FL-2 histogram from the FL-3 negative controls (not shown). See main text for discussion of results.

antibody binding capacity is derived from covalently bound goat anti-mouse immunoglobulin on the microbead. 50 $\mu$ l of solution containing approximately 100,000 microbeads were dispensed and labelled with directly conjugated 8G12-RPE using the standard protocol, with the exception that the incubation step with human gamma globulin was omitted (Table 3.13), and the mAb incubation was prolonged to 1hr (as recommended by the manufacturer). Similar amounts of mAb were used for microbead labelling as for cell labelling in order to ensure that saturation of binding sites occurred. Contemporaneous KG1a samples were stained with 8G12-RPE and IgG1-RPE. Acquisition was carried out with the same cytometer set-up (Table 3.12). Microbeads are approximately the size of lymphocytes, and were acquired through gating on the singlet population (Figure 3.14), whilst KG1a were acquired through their usual gate. The peak arithmetic channel numbers for each of the five microbead populations were recorded, a correlation coefficient and regression line was calculated, and a calibration curve of the arithmetic histogram channel (abscissa) against the antibody binding capacity (ordinate) was plotted (Figure 3.15). In this experiment the correlation coefficient was  $r = +1.00$ , and the regression line was described by the formula  $y = -1,798 + 618.x$ . The mean fluorescence intensity of 8G12-RPE staining of KG1a could then be used to estimate the mean number of antibody binding sites present on KG1a by calculation from the regression equation or by extrapolation from the calibration graph. Mean KG1a fluorescence in the negative control was 2.46, equating to an antibody binding capacity of -277 (an artefact of the formula), whilst mean fluorescence in the positive control was 1,596, equating to an antibody binding capacity of 984,530. This is equivalent to the number of cognate epitopes expressed by KG1a. A Coulter Channalyser 256 measured a KG1a diameter of 10.8 $\mu$ m, giving a calculated surface area of 366.4 $\mu$ m<sup>2</sup> (based on the inaccurate assumption that cells are spherical) and an epitope density of 2,687/ $\mu$ m<sup>2</sup>.

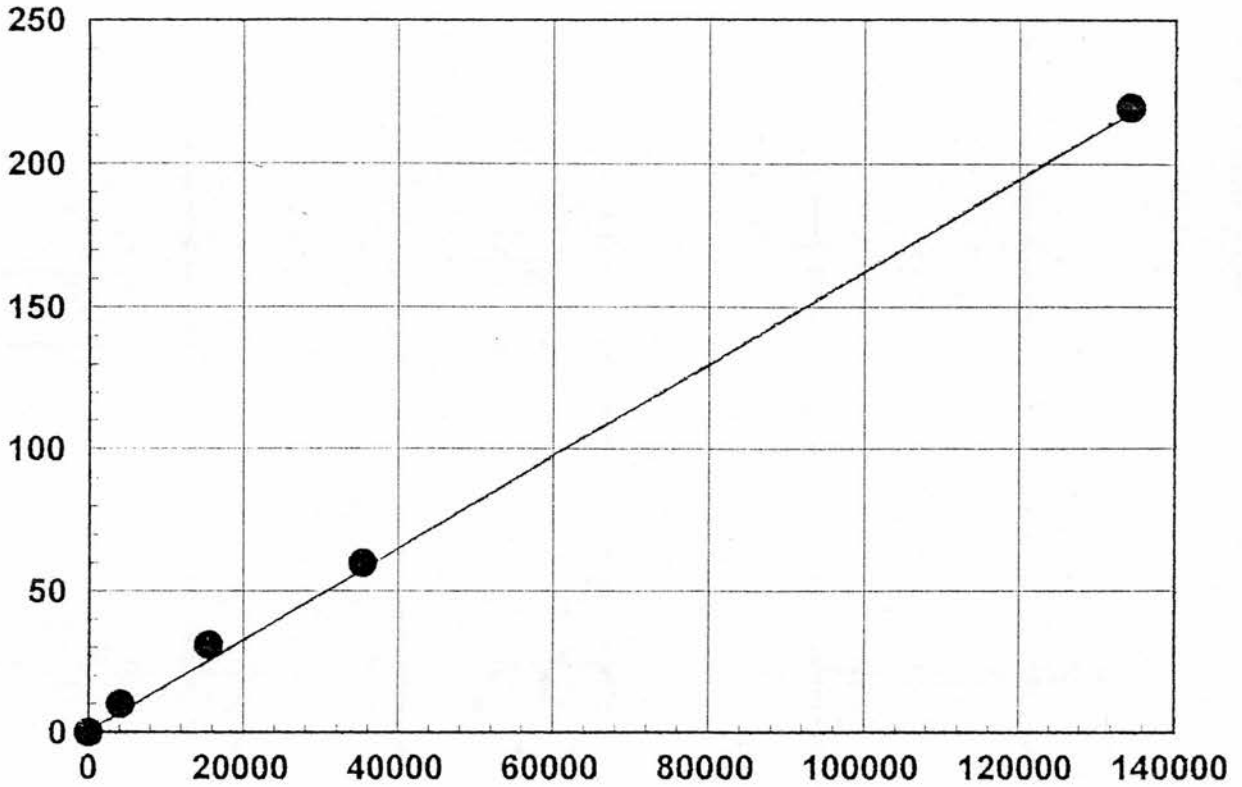
A second experiment was carried out in which microbeads were again labelled with 8G12-RPE, whilst a normal bone marrow sample was labelled with 8G12-RPE and a CD45-FITC conjugate. The samples were acquired using the normal

**Figure 3.14 Physical Characteristics and Fluorescence of Microbeads.**



*Legend.* An acquisition gate was established on the singlet population of 8G12-RPE labelled microbeads on the FSC / SSC dot-plot (A & B), allowing discrimination of five discrete populations on the FL-2 histogram (C). These demonstrated peak channel numbers of M1: 2.21, M2: 15.26, M3: 50.94, M4: 113 and M5: 375 respectively (C). This data was used to construct a calibration curve of peak channel number against antibody binding sites as demonstrated in Figure 3.15 and discussed in the main text.

Figure 3.15 Calibration Curve Established using Microbeads.



*Legend.* The peak channel numbers for each of the 5 populations of 8G12-RPE labelled microbeads were recorded (Figure 3.14) and plotted against the known mAb binding capacity. The correlation coefficient was  $r = +1.00$  and the regression line was described by the formula  $y = -1,798 + 618.x$ . *Abcissa:* mAb binding capacity. *Ordinate:* peak channel number. The solid data points are those observed. Too a large extent these overlap. Data is tabulated in Appendix 3 Table A13.



cytometer set-up (Table 2.6). Microbeads were acquired through a gate on the singlet population as previously, whilst the bone marrow samples were acquired through a FSC / SSC gate excluding debris and aggregates. In this experiment the correlation coefficient was (once again)  $r = +1.00$ , and the regression line was described by the formula  $y = -2,452 + 362.x$ . The peak channel number for 8G12-RPE staining of the  $CD34^+$  population was 152, corresponding to an antibody binding capacity of 52,588. An average lymphocyte diameter of  $7.2\mu\text{m}$ , gives a calculated surface area of  $162.9\mu\text{m}^2$  and an estimated epitope density of  $323/\mu\text{m}^2$ . This study does not prove that CD34 antigen density is greater on KG1a than HPC. It is possible that KG1a express a different form of CD34 with more epitopes per molecule, or that the structure of the membrane is different and provides a greater effective surface area (cells are not spheres). Nevertheless this kind of calculation is useful in providing a measure of antigen density on comparable cells, and demonstrates one of the reasons why  $CD34^+$  HPC are more difficult to define than  $CD34^+$  KG1a.

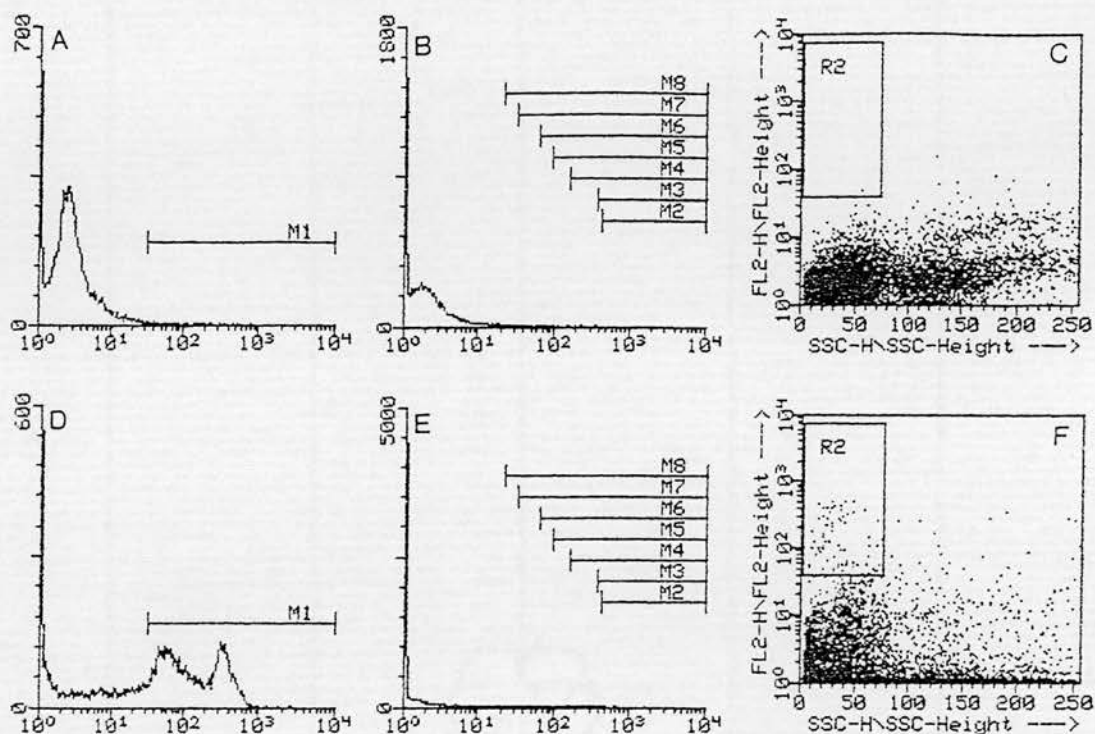
A third series of experiments were carried out in which KG1a and microbeads were stained with anti-CD44 mAb and with RAM-PerCP as a second step reagent. A control set of beads was stained with the second step reagent alone. Unfortunately discrimination of the separate populations of beads was poor using this indirect technique. This is perhaps not surprising because the variable orientation of the primary mAb on the surface of the beads, binding of the second step reagent directly by the beads and the variable number of RAM-PerCP molecules which may bind to an individual primary mAb, all conspire to degrade the linearity of the relationship between the number of binding sites presented and the intensity of fluorescence.

### *3.5.3 Contamination of the study population with variation in the discriminatory marker.*

If cells were to be acquired on the basis of  $CD34^+$ , it was important that the acquisition was carried out in such a way as to provide a measure of the level of contamination. In an initial series of experiments HPC were acquired on the basis of

CD34<sup>+</sup> alone, defined on the basis of the FL-2 negative control acquired through an open gate. The acquired cells proved to give rise to considerable spectral overlap into both FL-1 and FL-3. On closer examination it was found that highly FL-2 autofluorescent cells, which provided the main false positive contamination of the study population, also provided most of the spectral overlap in the other channels. Furthermore, much of the autofluorescent contamination was found to be arising from the debris and cell aggregates. It was clear, therefore, that the study population would have to be acquired through a more complex gate on FSC and FL-2. A somewhat involved set-up procedure was adopted: CD34-RPE / CD45-FITC labelled cells and the appropriate negative controls were used to establish an acquisition gate by CD45 backgating as described in Chapter 2.4.5, except that debris and cell aggregates were excluded on the FSC alone rather than on the FSC-SSC dot-plot. A series of markers were established on the negative control acquired through this gate, and the percentages compared with those on the CD34<sup>+</sup> sample in order to define the placement of the discriminatory marker. This analysis was quite informative in that it demonstrated that the higher the discriminatory marker the lower the contamination of the CD34<sup>+</sup> population (as expected), but also that the CD34<sup>+</sup> population itself has a broader range of fluorescence than suspected (Figure 3.16). 0.05% was chosen as the discriminatory marker because a higher marker engenders greater contamination of the study population (as discussed in Chapter 2.9.3) and also therefore obscuration of the analysis of the study population due to greater spectral overlap, but it has to be acknowledged that this biases the data in favour of the more primitive CD34<sup>hi</sup> population. A similar type of problem arises when enrichment of HPC is carried out on the basis of CD34 positivity (Chapter 6.6). A 0.05% discriminatory marker defined on analysis of the negative control gated on FSC alone was therefore used to backgate the CD34<sup>+</sup> population onto the FSC / FL-2 dot-plot such that an acquisition gate could be established on these two parameters (Figure 3.17). A minor adjustment to the compensation in FL-1 and FL3 was then necessary (Table 3.14).

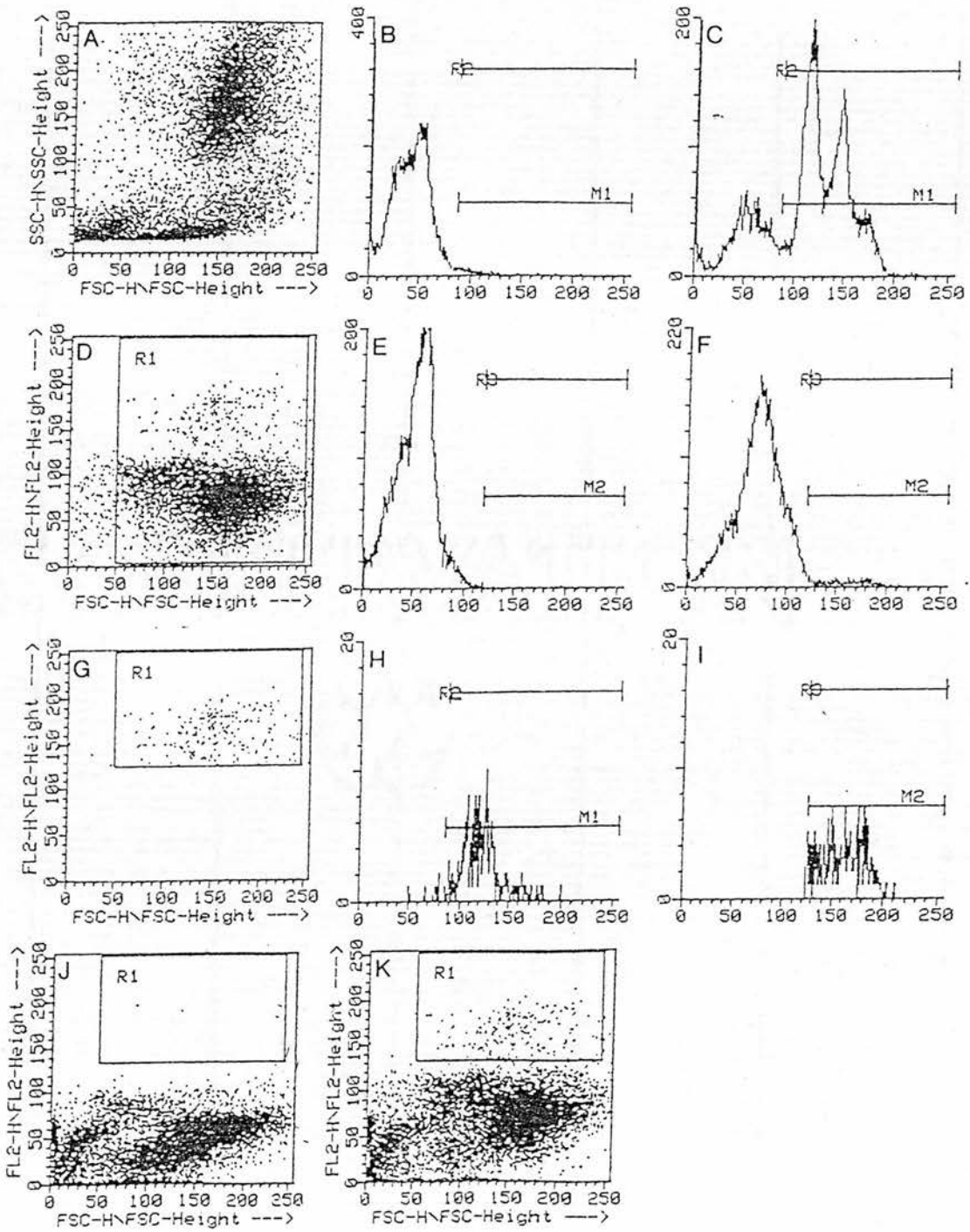
**Figure 3.16 Range of Fluorescence of the CD34<sup>+</sup> Population and Dependence of Contamination of the Study Population on the Discriminatory Marker.**



*Legend.* A series of markers were established on the negative control (A, B & C) and on PB<sup>m</sup> labelled with CD45-FITC and CD34-RPE (D, E & F).

FL-1 M1: (A) 1.0% (D) 61.6%. FL-2 M2: (B) 0.00% (E) 0.08% contamination <12.5%. FL-2 M3: (B) 0.01% (E) 0.12% contamination 8.3%. FL-2 M4: (B) 0.05% (E) 0.44% contamination 11.4%. FL-2 M5: (B) 0.10% (E) 0.65% contamination 16.7%. FL-2 M6: (B) 0.20% (E) 0.81% contamination 24.7%. FL-2 M7: (B) 0.50% (E) 1.43% contamination 36.4%. FL-2 M8: (B) 0.94% (E) 2.05% contamination 45.8%. It is clear that CD34<sup>+</sup> cells overlap with the negative population over a wide range of fluorescence. The maximal CD34<sup>+</sup> count (after subtraction of false positivity) is achieved at M8 (1.11%) and minimal contamination at M2 or M3. The use of the SSC / FL-2 dot-plot allows better discrimination of false positives R2 (C) 0.01% from CD34<sup>+</sup> cells R2 (F) 0.83%. Contamination was 1.2% due to exclusion of high side scatter cells.

Figure 3.17 Establishment of an Acquisition Gate on the FSC / FL-2 Dot-Plot.



*Legend.* Negative control and CD45-FITC, CD34-RPE labelled samples from PB<sup>m</sup> were acquired through an open gate (A) and used to establish a marker on FL-1: negative control (B) M1: 1.44%, CD45-FITC labelled sample (C) M1: 73.97%. The CD45<sup>+</sup> cells were backgated through region R2 (=M1) onto the FSC / FL-2 dot-plot (D), and a region R1 was defined to exclude FSC<sup>lo</sup> CD45<sup>-</sup> debris and FSC<sup>hi</sup> aggregates. Cells acquired through this gate were used to establish a marker on FL-2 negative control (E) M2: 0.05% and CD34-RPE labelled sample (F) M2: 3.08%. The CD34<sup>+</sup> cells were backgated through region R3 (=M2) onto the FSC / FL-2 dot-plot (G) allowing adjustment of the acquisition gate R1. Cells acquired through the adjusted R1 gate demonstrated a CD45<sup>+</sup> of 96.8% (H) and a CD34<sup>+</sup> of 100% (I). Test samples acquired through an open gate demonstrated that 0.02% of the negative control (J) and 1.94% of the CD34<sup>+</sup> labelled sample (K) would fall into R1, leading to a calculated contamination of 1%.

**Table 3.14 Flow Cytometer Settings for Three-Colour Immunocytometry.**

<i>Parameter</i>	<i>Detector</i>	<i>Amplification</i>	<i>Threshold</i>
FSC	E00	1.30	52
SSC	340	1.00	
FL-1	575	Log	
FL-2	565	Log	
FL-3	600	Log	
<i>Compensation</i>			
FL1 - %FL2	1.7%		
FL2 - %FL1	42.6%		
FL2 - %FL3	0.0%		
FL3 - %FL2	10.0%		

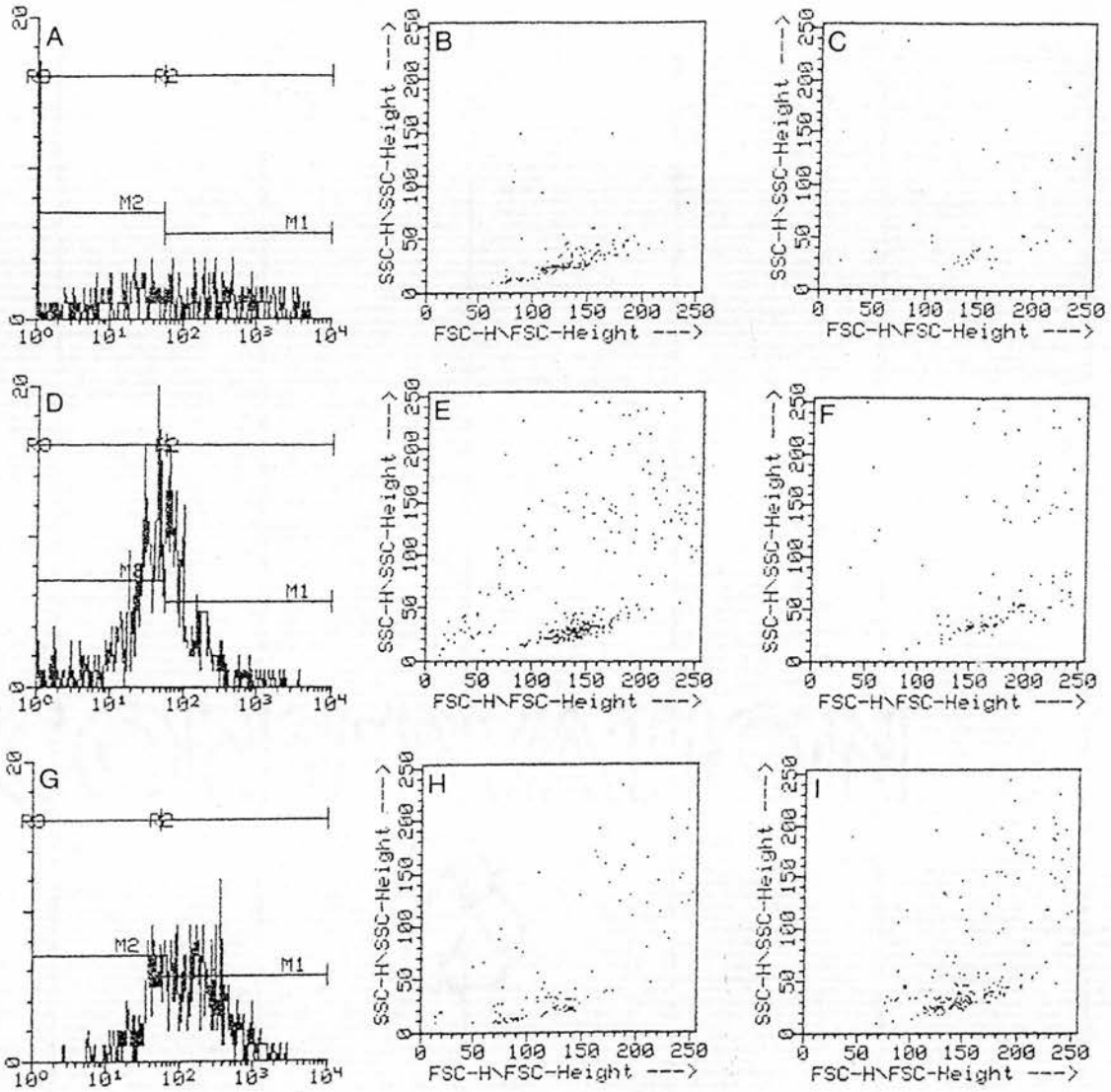


#### 3.5.4 Discrimination of CD34<sup>+</sup> lineage subsets by light-scatter.

Since, the intention of this study was to quantify the differences in CAM expression between HPC subsets, it was thought valuable to determine whether CD34<sup>+</sup> subsets could be resolved on the basis of light scatter as well as antigen expression. Samples stained for anti-CD34 and lineage markers were acquired using the CD45 backgating technique described above (Chapter 2.4.5). During analysis a discriminatory marker established on the CD34<sup>+</sup> IgG-FITC was used to discriminate fluorescence positive and negative subpopulations and to project these back onto the FSC / SSC dot-plot. It became obvious that there were simply too few CD34<sup>+</sup> CD33<sup>+</sup>, CD34<sup>+</sup> CD7<sup>+</sup>, CD34<sup>+</sup> CD19<sup>+</sup> or CD34<sup>+</sup> CD41a<sup>+</sup> cells available to achieve useful information on distribution. In general, the CD34<sup>+</sup> CD71<sup>+</sup>, CD34<sup>+</sup> HLA-DR<sup>+</sup> and (to a lesser extent) CD34<sup>+</sup> CD38<sup>+</sup> subpopulations appeared to contain more high forward and side scattering cells than their respective negative populations (Figure 3.18). These light scatter characteristics are consistent with studies using counterflow elutriation which demonstrate that CD34<sup>hi</sup> HLA-DR<sup>-</sup> and CD38<sup>-</sup> populations consist of small, low density early progenitors and stem cells, whilst CD34<sup>lo</sup> HLA-DR<sup>+</sup> and CD38<sup>+</sup> cells are larger, denser, committed HPC [Herbein, Sovalat, Wunder *et al.* 1994].

Terstappen *et al* have demonstrated that the physical characteristics of granular and non-granular lymphocytes cannot be resolved using standard linear or logarithmic functions, but can be separated easily if the side scatter data is transformed by a polynomial function so as to increase the resolution in the middle of the SSC range [Terstappen, Mickaels, Dost *et al.* 1990]. A similar analytical programme applied to data on HPC subset distribution may be more informative than the standard software. Nevertheless this information provided a further argument for acquiring data on the basis of a FSC / CD34<sup>+</sup> gate, and not excluding CD34<sup>+</sup> SSC<sup>hi</sup> cells from the analysis.

Figure 3.18 Light Scatter Characteristics of CD34<sup>+</sup> Subsets.



*Legend.* Cells from a leucapheresis product were acquired on a gate (R1) established on CD34<sup>+</sup> alone. Negative (M2 = R3) and positive (M1 = R2) markers and gates were established on the negative control (not shown) and were used to backgate the CD34<sup>+</sup> Marker<sup>-</sup> and CD34<sup>+</sup> Marker<sup>+</sup> populations onto the FSC / SSC dot-plots. (A) CD34<sup>+</sup> CD71 (FL-1) histogram. (B) CD34<sup>+</sup> CD71<sup>-</sup> FSC / SSC dot-plot (C) CD34<sup>+</sup> CD71<sup>+</sup> FSC / SSC dot-plot (D) CD34<sup>+</sup> HLA-DR (FL-1) histogram. (E) CD34<sup>+</sup> HLA-DR<sup>-</sup> FSC / SSC dot-plot (F) CD34<sup>+</sup> HLA-DR<sup>+</sup> FSC / SSC dot-plot. (G) CD34<sup>+</sup> CD38 (FL-1) histogram. (H) CD34<sup>+</sup> CD38<sup>-</sup> FSC / SSC dot-plot (I) CD34<sup>+</sup> CD38<sup>+</sup> FSC / SSC dot-plot.

### 3.5.5 Three colour analysis of adhesion molecule expression within bone marrow HPC subsets.

In view of the difficulties encountered in resolving CD34<sup>+</sup> lineage subsets described above it was decided that a more modest objective would be established: to determine whether LFA-3 and VLA-5 expression varied with CD71, HLA-DR and CD38 expression. This seemed reasonable in that it was unlikely that the presence or absence of minor subpopulations would contribute to significant shifts in CAM expression, and because all three of the activation markers demonstrated higher expression in sessile compared to circulating HPC (Section 3.3.2). The labelling protocol is described in Table 3.13, the cytometer set-up in Table 3.14 and the panel of assays in Table 3.15. An IgG2a mAb to CD44 and an irrelevant-specificity IgG2a were used as positive and negative controls respectively because the LFA-3 and VLA-5 mAbs were IgG2a isotypes. A FSC / FL-2 acquisition gate was established using the negative control (sample #15) and a positive control (sample #13) as previously described (Section 3.5.3 and 3.5.4). Under an open gate 10,000 events were acquired in order to establish the CD34%<sup>+</sup> and the %fl<sup>+</sup> of the negative control, from which the level of contamination of the study population could be estimated. The acquisition gate was further adjusted to ensure there was no negative tail on the study population (Section 3.5.3). A target of 2,000 events was acquired through this gate, necessitating evaluation of up to 500,000 cells per sample. Discrimination of activation positive and negative CD34<sup>+</sup> cells was carried out by analysis of the CD34<sup>+</sup> FL-1 histogram on samples #13 and #14 as previously described (Section 3.3). Analysis of CAM expression within CD34<sup>+</sup> lin<sup>+</sup> and lin<sup>-</sup> subpopulations was carried out by establishing discriminatory markers on the FL-3 histogram using the relevant positive and negative controls (*e.g.* #3 and #4 for the CD34<sup>+</sup> CD71-labelled samples) and the mfi of FL-3 antigen expression (Figure 3.19) [Terstappen, Hollander, Meiners *et al*, 1990]. Antigen quantitation could not be carried out because of the failure of the calibration beads with indirect technique as described above (Section 3.5.2). The results were compared to the relevant negative controls and to each other using paired *t* tests (Tables 3.16-3.18).

**Table 3.15 Test Panel for the Three Colour Immunocytometry Study.**

#1. CD58 RAM-Biotin Strep-QR	CD71-FITC	CD34-RPE
#2 CD49d RAM-Biotin Strep-QR	CD71-FITC	CD34-RPE
#3 CD44 RAM-Biotin Strep-QR	CD71-FITC	CD34-RPE
#4 IgG2a RAM-Biotin Strep-QR	CD71-FITC	CD34-RPE
#5. CD58 RAM-Biotin Strep-QR	CD38-FITC	CD34-RPE
#6 CD49d RAM-Biotin Strep-QR	CD38-FITC	CD34-RPE
#7 CD44 RAM-Biotin Strep-QR	CD38-FITC	CD34-RPE
#8 IgG2a RAM-Biotin Strep-QR	CD38-FITC	CD34-RPE
#9. CD58 RAM-Biotin Strep-QR	HLA-DR-FITC	CD34-RPE
#10 CD49d RAM-Biotin Strep-QR	HLA-DR-FITC	CD34-RPE
#11. CD44 RAM-Biotin Strep-QR	HLA-DR-FITC	CD34-RPE
#12 IgG2a RAM-Biotin Strep-QR	HLA-DR-FITC	CD34-RPE
#13. CD45-FITC	CD34-RPE	
#14. IgG1-FITC	CD34-FITC	
#15. IgG1-FITC	IgG1-RPE.	
#16 autofluorescence.		

*Legend.* CD34-RPE: anti-CD34 mAb directly conjugated with RPE.

CD\*\*-FITC: mAb addressed to a lineage or activation marker directly conjugated to FITC. SAM-FITC: sheep anti-mouse antibody conjugated to FITC. CD\*\*: purified anti-cell adhesion molecule mAb. RAM-Biotin Strep-QR: biotinylated rat anti-mouse and streptavidin-linked Quantum Red. Labelled samples were compared with similarly conjugated mAbs of the same isotype but irrelevant specificity.

There were found to be two problems in the interpretation of the results: First, a relatively wide standard deviation such that it was difficult to statistically discriminate LFA-3 (CD58) and VLA-5 (CD49e) expression from the negative control (CD44 expression could be statistically discriminated in the main) (Tables 3.16 & 3.17). Second, there was a clear difference in negative control fluorescence between lineage subsets with increasing background in the general order  $CD34^+ \text{ lin}^- < CD34^+ \text{ global} < CD34^+ \text{ lin}^+$  (Figure 3.19 & Table 3.18). Allowing for these limitations, LFA-3 (CD58) did not vary significantly between subsets whilst VLA-5 (CD49e) expression did appear to be greater in the  $CD34^+ \text{ CD71}^+$  and  $CD34^+ \text{ CD38}^+$  subpopulations compared to their activation negative counterparts (Table 3.18).

### 3.6 Discussion.

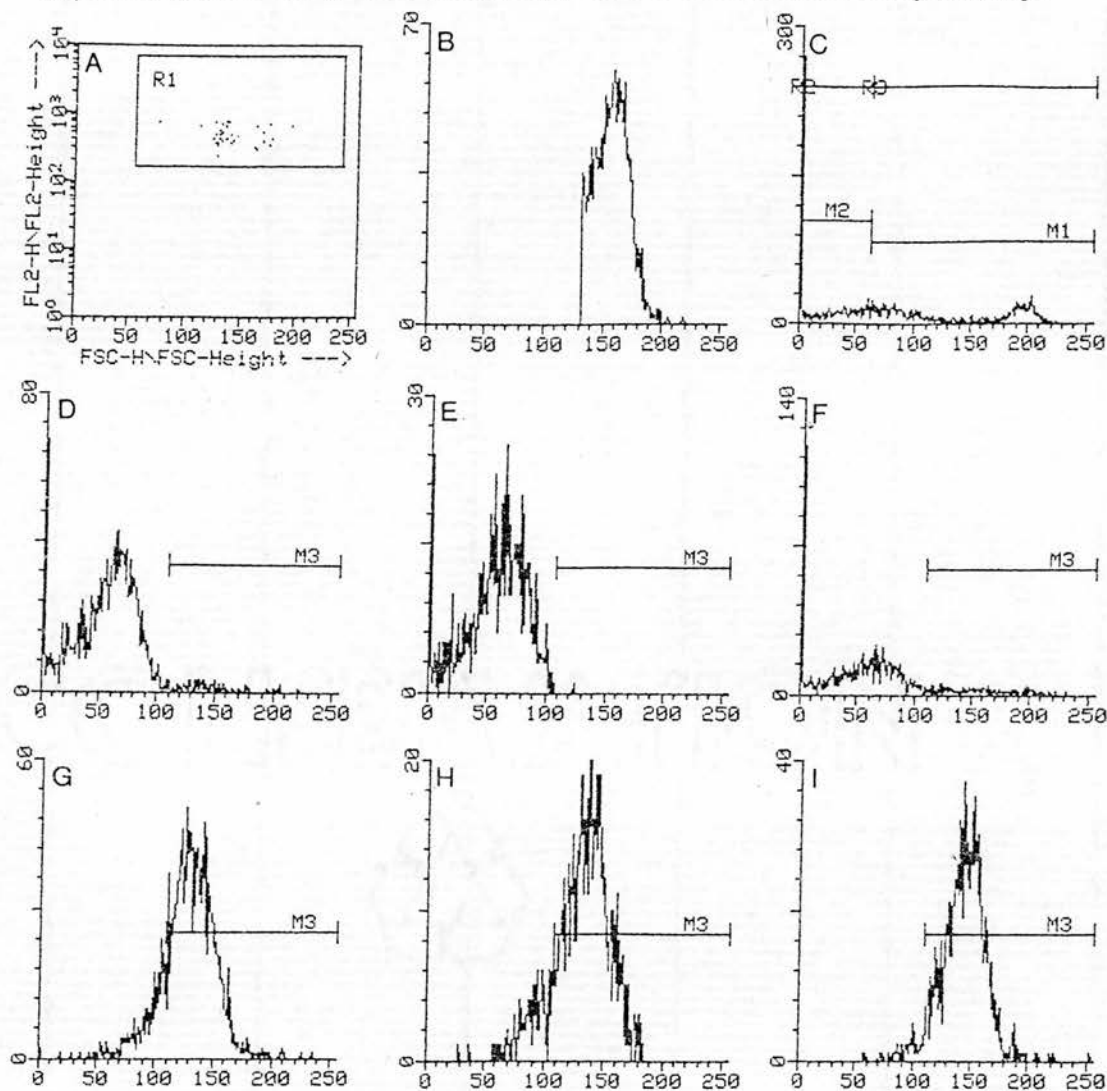
The work described in this Chapter aimed to clarify the pattern of cell adhesion molecule expression by HPC and demonstrated that differences occur between sessile and circulating populations. The observation that there are source dependent differences in HPC lineage and activation subsets was confirmed. Although cell line studies suggest that differences in CAM expression and lineage commitment may be linked, it proved very difficult to test this proposition on HPC because of the small size of the study population and a wide standard deviation of most measurements of antigen expression. The precision and accuracy of the immunocytometry technique improved over the series of studies detailed as a reflection of improvements in reagents and labelling and in analytical methodology. Nevertheless, some important problems remain.

#### *3.6.1 Problems associated with the interpretation of adhesion molecule expression.*

The data on HPC adhesion molecule expression was summarised in Section 3.2.3 and Figures 3.2 and 3.3, and the problems associated with interpreting the validity of comparative data or the physiological relevance of the observations is discussed in Section 3.2.5. The results on BM  $CD34^+$  adhesion molecule expression are broadly comparable with those of other groups [Lewinsohn, Nagler, Ginzton *et*



**Figure 3.19 Analysis of Cell Adhesion Molecule Expression within Haematopoietic Progenitor Subsets by Three Colour Immunocytometry.**



*Legend.* Cells were acquired through an acquisition gate (R1) established on the FSC / FL-2 dot-plot (A) as previously described (Figure 3.17). The study population was CD34<sup>+</sup> on FL-2 (B) and CD71<sup>-</sup> (M2 = R2) and CD71<sup>+</sup> (M1 = R3) populations were defined on the FL-1 histogram (C) using the appropriate negative controls (Figure 3.5). CD34<sup>+</sup> IgG2a (D) and CD34<sup>+</sup> CD44<sup>+</sup> (G) were used to define positivity in FL-3. Expression of the third antigen within the CD34<sup>+</sup> CD71<sup>-</sup> (R2) population (E & H) and the CD34<sup>+</sup> CD71<sup>+</sup> (R3) population (F & I) could then be compared to that within the CD34<sup>+</sup> population as a whole (D & G) using percentage positivity and mean fluorescence intensity.



**Table 3.16 Percentage Positive CAM Fluorescence within CD34<sup>+</sup> Lin<sup>+</sup> and Lin<sup>-</sup> Subsets.**

<i>phenotype</i>	<i>CD34<sup>+</sup> FL-3<sup>+</sup></i>	<i>CD34<sup>+</sup> FL-I<sup>-</sup> FL-3<sup>+</sup></i>	<i>CD34<sup>+</sup> FL-I<sup>+</sup> FL-3<sup>+</sup></i>
CD34 71 58.	13.8 ± 9.5% ns	0.7 ± 0.7% ns	31.8 ± 24.3% ns
CD34 71 49e.	24.7 ± 21.7% ns	18.9 ± 19.1% ns	35.9 ± 26.2% ns
CD34 71 IgG.	7.7 ± 4.2%	0.16 ± 0.17%	18.6 ± 12.5%
CD34 71 CD44.	91.6 ± 5.0% p<0.001	87.2 ± 8.8% p<0.001	95.2 ± 3.4% p<0.001
CD34 38 58.	7.1 ± 4.8% ns	0.8 ± 0.9% ns	10.6 ± 7.8% ns
CD34 38 49e.	23.7 ± 20.1% ns	12.6 ± 17.4% ns	29.8 ± 21.8% ns
CD34 38 IgG.	6.6 ± 4.6%	0.85 ± 1.25%	9.9 ± 7.0%
CD34 38 44.	88.9 ± 9.7% p<0.001	93.9 ± 4.7% p<0.001	87.6 ± 10.5% p<0.001
CD34 HLADR 58.	7.2 ± 4.3% ns	1.1 ± 0.9% ns	9.2 ± 6.2% ns
CD34 HLADR 49e.	22.0 ± 18.8% ns	13.6 ± 11.5% ns	26.6 ± 22.3% ns
CD34 HLADR IgG.	5.7 ± 3.3%	0.8 ± 0.2%	7.3 ± 4.4%
CD34 HLADR 44.	88.0 ± 10.0% p<0.001	78.5 ± 18.9% p<0.01	89.0 ± 10.1% p<0.001

**Table 3.17 Mean Fluorescence Intensity of CAM Expression within CD34<sup>+</sup> Lin<sup>+</sup> and Lin<sup>-</sup> Subsets.**

<i>phenotype</i>	<i>CD34<sup>+</sup> FL-3<sup>+</sup></i>	<i>CD34<sup>+</sup> FL-1<sup>-</sup> FL-3<sup>+</sup></i>	<i>CD34<sup>+</sup> FL-1<sup>+</sup> FL-3<sup>+</sup></i>
CD34 71 58.	86 ± 29 ns	36 ± 21 ns	162 ± 120 ns
CD34 71 49e.	93 ± 36 ns	75 ± 31 ns	117 ± 39 ns
CD34 71 IgG.	61 ± 31	29 ± 26	100 ± 31
CD34 71 CD44.	250 ± 69 p<0.05	210 ± 52 p<0.02	301 ± 103 p<0.05
CD34 38 58.	58 ± 31 ns	34 ± 20 ns	70 ± 32 ns
CD34 38 49e.	89 ± 28 ns	67 ± 22 ns	101 ± 24 ns
CD34 38 IgG.	56 ± 21	34 ± 20	68 ± 22
CD34, 38 44.	256 ± 83 p<0.05	256 ± 83 p<0.05	312 ± 145 p<0.05
CD34 HLADR 58.	57 ± 26 ns	49 ± 54 ns	64 ± 25 ns
CD34 HLADR 49e.	79 ± 24 ns	62 ± 24 ns	85 ± 25 ns
CD34 HLADR IgG.	48 ± 28	30 ± 21	52 ± 27
CD34 HLADR 44.	276 ± 142 ns	260 ± 99 p<0.05	288 ± 165 ns

*Legend.* In the preceding tables results of %fl<sup>+</sup> and mfi of CAM expression within the CD34<sup>+</sup> population as a whole and within the CD34<sup>+</sup> lin<sup>-</sup> and lin<sup>+</sup> populations are reported as the mean ± standard deviation of 4 experiments, and are compared to the relevant negative controls by paired *t* test. Discussion of results - see main text.

**Table 3.18 Statistical Analysis of CAM Expression within CD34<sup>+</sup> Lin<sup>-</sup> and Lin<sup>+</sup> Subsets.**

<i>phenotype</i>	<i>global:lin<sup>-</sup></i>		<i>global:lin<sup>+</sup></i>		<i>lin<sup>-</sup>:lin<sup>+</sup></i>	
	<i>%fl<sup>+</sup></i>	<i>mfi</i>	<i>%fl<sup>+</sup></i>	<i>mfi</i>	<i>%fl<sup>+</sup></i>	<i>mfi</i>
CD34 71 58.	ns	ns	ns	ns	ns	ns
CD34 71 49e.	p<0.05	p<0.01	p<0.05	ns	p<0.05	p<0.02
CD34 71 IgG.	ns	p<0.01	ns	ns	ns	p<0.05
CD34 71 CD44.	ns	ns	p<0.05	ns	ns	ns
CD34 38 58.	ns	ns	ns	ns	ns	ns
CD34 38 49e.	p<0.01	ns	ns	ns	p<0.01	ns
CD34 38 IgG.	p<0.05	p<0.02	ns	ns	ns	p<0.05
CD34 38 44.	ns	ns	p<0.05	ns	ns	ns
CD34 HLADR 58.	ns	ns	ns	ns	ns	ns
CD34 HLADR 49e.	ns	ns	ns	ns	ns	ns
CD34 HLADR IgG.	p<0.05	ns	ns	ns	ns	p<0.05
CD34 HLADR 44.	ns	ns	p<0.02	ns	ns	ns

*Legend.* Results of %fl<sup>+</sup> and mfi of CAM expression are compared using paired *t* tests: CD34<sup>+</sup> population with CD34<sup>+</sup> lin<sup>-</sup> population (*global:lin<sup>-</sup>*), CD34<sup>+</sup> population with CD34<sup>+</sup> lin<sup>+</sup> population (*global:lin<sup>+</sup>*) and CD34<sup>+</sup> lin<sup>-</sup> population with CD34<sup>+</sup> lin<sup>+</sup> population (*lin<sup>-</sup>:lin<sup>+</sup>*). For discussion of results - see main text.

*al.* 1990; Long & Dixit, 1990; Soligo, Schiró, Luksch *et al.* 1990; Arkin, Naprstek, Guarini *et al.* 1991; Saeland, Duvert, Caux *et al.* 1992; Greenwalt, Lipsky, Ockenhouse *et al.* 1992; Simmons, Niutta, Ashman *et al.* 1992; Watt, Williamson, Genevieve *et al.* 1993; Liesveld, Winslow, Frediani *et al.* 1993], and the implications for a model of HPC homing and engraftment are discussed in Chapter 7.2.

There are few studies which directly compare CAM expression between BM and circulating CD34<sup>+</sup> cells. Möhle, Haas & Hunstein, [1993] reported LFA-1 to be significantly reduced in LP HPC from 23 patients, 15 mobilised with cytotoxic chemotherapy and rhu-G- CSF, and 8 with a combination of rhuIL-3 and rhuGM-CSF. No difference in ICAM-1 or CD44 expression was demonstrated, and no other CAMs were studied. Leavesley, Oliver, Swart *et al* [1994], have reported reduced expression of LFA-1 and VLA-4, but not PECAM-1 in cryopreserved LP HPC from 3 patients following chemotherapy and rhuG-CSF. Again no other CAMs were studied. Saeland, Duvert, Caux *et al* [1992], found no difference in the expression of adhesion molecules between BM and UCB CD34<sup>+</sup> cells using a semiquantitative reporting system.

The discordance between the findings in these studies and those in the present study may relate to several factors including differences in the study population (*e.g.* cytokine exposure or cryopreservation), variation in immunofluorescence labelling and cytometry technique (discussed further below in Section 3.7.2), and stochastic variation between studies.

The power of a study is dependent on the size of the study population, the variation in the data (SD), the size of difference sought, and the level of statistical significance demanded [Altman, 1994]. For example, calculating from the data presented in Table A4, the power of this study to detect a difference of 15% in LFA-1 expression between BM and LP CD34<sup>+</sup> cells, given a SD of 12.5% (a calculated standard difference of 1.2) and n=8, was approximately 0.20 for a p value of <0.01, and 0.40 for a p value of <0.05. In other words, there was an 80% chance of obtaining a non-significant result when a 15% difference in LFA-1 expression between BM and LP CD34<sup>+</sup> cells did in fact exist (a type II error) allowing for a p



value of  $<0.01$ , and a 60% chance allowing for a p value of  $<0.05$ . In the study by Mohle, Haas & Hunstein [1993], a 15% difference in LFA-1 expression between BM and LP CD34<sup>+</sup> cells was detected at a  $p<0.001$  (my calculation based on their published data), though the power of their study is difficult to estimate because of inequality in the size of the study populations (BM  $n=6$ , LP  $n=23$ ). In general, the larger the comparative study population, and the smaller the variation in expression of the antigen of interest, the greater the power of the study. There is a strong argument therefore for carrying out highly focused studies with large study populations. Given an SD of 12.5%, in order to achieve a 95% chance of detecting a difference of 10% between BM and LP CD34<sup>+</sup> CAM expression (at a p value of  $<0.01$ ), it would be necessary to study at least 100 paired samples.

### *3.6.2 Problems associated with the definition of HPC-subsets.*

Caution was exercised in the interpretation of antigen expression as a marker of lineage-committment. First, quantitative multifactorial analysis of peripheral blood leucocyte populations has illustrated that antigen expression is rarely lineage-restricted and is better described as lineage-associated [Terstappen, Hollander, Meiners *et al*, 1990]. In particular, considerable uncertainty exists as to the pattern of expression of lineage-associated antigens on earlier progenitors. It has been proposed that some observations of "lineage- infidelity" in leukaemic phenotypes, may be representative of a general phase of "lineage-promiscuity" amongst multipotential cells, during which lineage-specific antigens may be coexpressed prior to irreversible committment [Greaves, Chan, Furley *et al*. 1986; McCulloch, 1987]. Recent advances in immunocytometric technique provide support for this thesis [Hurwitz, Gore, Stone *et al*. 1992]. Second, considerable variability exists between the reports from different groups on the levels of antigen positivity or expression. This is related to two phenomena: first, an absence of standardisation between research groups with regard to the nature of the study populations, the preparatory handling (including enrichment techniques), the epitope specificity and fluorescence intensity of the mAb-fluorochrome conjugates (see for example

Chapter 2.4.6), labelling technique, cytometer set-up and acquisition gates (Chapter 2.6), and analytical criteria including definition of the CD34<sup>+</sup> population and of lineage marker positivity (Chapter 2.9.3). Second, stochastic variation occurs between studies and this is particularly marked if the study population is small (Chapters 2.7 and 2.9.2).

CD33 expression is thought to be restricted to myeloid lineage cells [Andrews, Singer & Bernstein, 1990; Terstappen, Hollander, Meiners *et al*, 1990; Terstappen & Loken, 1990]. Overall, CD34<sup>+</sup> CD33 expression was very low in this study despite detection of the antigen in the BM CD34<sup>+</sup> population (Figure 3.5 - 3.7). These results are comparable with those of Fritsch, Buchinger, Printz *et al*. [1993a], who found CD34<sup>+</sup> cells to be predominantly CD33<sup>-</sup> or <sup>lo</sup>. In contrast many groups have described significant CD34<sup>+</sup> CD33 expression: Bender *et al* reported higher levels of CD33 expression by PB<sup>m</sup> (73.5%) and PB<sup>s</sup> (84%) CD34<sup>+</sup> cells compared to those in BM (43%) [Bender, Unverzagt, Walker *et al*. 1991; Bender, Williams, Myers *et al*, 1992]. CD33 antigen density was however described as low with some patients (30%) predominantly negative. Saeland, Duvert, Caux *et al*. [1992] reported CD34<sup>+</sup> CD33 expression at low antigen density and with considerable heterogeneity, leading to substantial overlap with the negative population. Mohle, Haas & Hunstein [1993] reported that 80% of PB<sup>m</sup> CD34<sup>+</sup> were CD33<sup>+</sup> albeit with substantial inter-individual variability. The consensus therefore seems to be that CD33 antigen expression within the CD34<sup>+</sup> population is low density and highly variable, leading to a wide variation in reported positivity. It is also possible that differences in epitope affinity or resolution index between mAbs are contributing to the problem. CD13 may be used as a marker of myeloid commitment, but Saeland, Duvert, Caux *et al* [1992] have reported broad expression of this antigen within the CD34<sup>+</sup> population including cells expressing lymphoid markers CD10<sup>+</sup> and CD19<sup>+</sup>.

Several groups have reported the presence of a significant CD34<sup>+</sup> subpopulation expressing B-lymphoid associated antigens (CD10, CD19 and CD20) in BM (6.5-10%) but not in CB or PB cells [Ryan, Kossover, Mitchell *et al*. 1986; Loken, Shah,



Dattilio *et al.*, 1987b; Bender, Unverzagt, Walker *et al.* 1991; Bender, Williams, Myers *et al.* 1992; Saeland, Duvert, Caux *et al.* 1992; Bender, Unverzagt, Walker *et al.* 1994; To, Haylock, Dowse *et al.* 1994]. CD19 displays exceptional B lineage-specificity, is amongst the earliest B-lymphoid antigens expressed, and is coexpressed with CD10, intranuclear TdT, and HLA-DR [Loken, Shah, Dattilio *et al.* 1987b; Serke, Sauberlich, Abe *et al.* 1991].

Terstappen, Huang & Pickler [1992] have demonstrated that CD34<sup>+</sup> thymocyte subsets in human thymus are HLA-DR, CD44 and CD54 low, CD38, CD7, CD49d and CD62L high. The same group were able to clearly demonstrate a CD34<sup>+</sup> CD7<sup>+</sup> population in foetal but not in adult BM. Saeland, Duvert, Caux *et al.* [1992] have confirmed virtually no expression of T-lymphoid associated antigens CD2, CD3, CD4, CD7 or CD8 on BM or CB HPC. Bender *et al.* have reported low level CD34<sup>+</sup> CD7 expression in BM (2.7%) and PB (7%), but not PB<sup>m</sup> [Bender, Unverzagt, Walker *et al.* 1991; Bender, Williams, Myers *et al.* 1992].

CD41a was not expressed by CD34<sup>+</sup> cells in this study, nor in that of Saeland, Duvert, Caux *et al.* [1992]. In comparison Bender, Williams, Myers *et al.* [1992] have reported high (81%) though heterogeneous expression of CD41 by PB<sup>m</sup> CD34<sup>+</sup> cells.

CD71 (transferrin receptor) was used as a marker of erythroid commitment in these studies, but is expressed by other proliferating cells [Loken, Shah, Dattilio *et al.* 1987a]. Expression of CD71 occurs in a subpopulation consisting of 40% of CD34<sup>+</sup> cells in BM, and slightly fewer in PB<sup>m</sup>. This result is similar to that of To, Haylock, Dowse *et al.* [1994], though Bender *et al.* [Bender, Unverzagt, Walker *et al.* 1991; Bender, Williams, Myers *et al.* 1992] reported a more polarised difference in CD71 expression between BM (94%) and PB (17%) and PB<sup>m</sup> (16%). Glycophorin A is widely used as a marker of erythroid differentiation, but is not expressed by HPC [Loken, Shah, Dattilio *et al.*, 1987a; Saeland, Duvert, Caux *et al.* 1992]. It may be better to use a mAb to the CD45RO isoform as a marker of primitive and erythroid HPC [Lansdorp, Sutherland & Eaves, 1990].

Both CD38 and HLA-DR are expressed by the majority of CD34<sup>+</sup> cells in a

homogeneous fashion (Figure 3.5 - 3.7 and Table 3.11), at significantly greater mfi in BM than in blood derived HPC, concordant with the observations of To, Haylock, Dowse *et al.* [1994]. Terstappen *et al* have reported that less than 1% of CD34<sup>+</sup> cells are CD38<sup>+</sup>, and that there is an inverse relationship between CD34 antigen intensity and CD38 antigen intensity, with the latter coexpressed with lineage- associated antigens CD71, CD33, CD10 or CD7 [Terstappen, Huang, Safford *et al.* 1991]. This group suggest that CD34<sup>hi</sup> CD38<sup>-</sup> lin<sup>-</sup> cells are a primitive blast population with characteristic intermediate FSC and low SSC, and that activation and lineage commitment is accompanied by decreasing CD34 expression, increasing CD38 and lineage-related antigen expression, and an increase in heterogeneity in the FSC and SSC characteristics of the population (Chapter 1.2.4 7 Section 3.5.4). HLA-DR is also expressed on the majority of CD34<sup>+</sup> cells, HLA-DR<sup>-</sup> populations being regarded as particularly primitive at least in adult bone marrow [Civin & Loken, 1987; Sutherland, Eaves, Eaves *et al.* 1989; Verfaillie, Blakholmer & McGlave, 1990; Srour, Brandt, Briddell *et al.* 1991; Huang & Terstappen, 1994] (Chapter 1.2.4).

In summary, differences in the state of activation / lineage commitment clearly do exist between BM-derived and PB-derived HPC, particularly with regard to the presence of a CD19<sup>+</sup> subset and a larger CD71<sup>+</sup> subset within BM, and greater homogeneous expression of CD38 and HLA-DR compared to PB, suggesting the presence of more activated CD34<sup>+</sup> population within the BM, with more erythroid and B-lymphoid precursors. Combination of lineage and activation markers using three colour immunocytometry may provide more accurate discrimination of CD34<sup>+</sup> subsets in the future.

### 3.6.3 Problems with three-colour immunocytometry of HPC subsets.

The difficulties in defining CD34<sup>+</sup> populations, quantitating CAM expression and identifying lineage subsets discussed in the preceding two chapters reach their apotheosis when attempting three-colour analysis. The central problem was to acquire sufficient cells to make an analysis meaningful. This was achieved by

acquiring through a gate established on FSC and CD34 positivity and analysing activation markers which showed a reasonable balance between positive and negative subpopulations. Fortuitously these markers were also those which showed differences between sessile and circulating CD34<sup>+</sup> populations *i.e.* CD71, CD38 and HLA-DR. In view of the extended nature of the labelling and analytical procedures it was decided to focus on the two CAMs which showed the most clear cut difference in expression between sessile and circulating HPC. Statistical variation between samples and a higher background fluorescence in the CD34<sup>+</sup> lin<sup>+</sup> subpopulation compared to the CD34<sup>+</sup> lin<sup>-</sup>, made interpretation very difficult. Allowing for this proviso, no difference in LFA-3 expression could be demonstrated between "activated" and "non-activated" CD34<sup>+</sup> populations, whilst higher expression of VLA-5 appeared to occur in the CD34<sup>+</sup> CD71<sup>+</sup> and CD34<sup>+</sup> CD38<sup>+</sup> subpopulations compared to the relevant CD34<sup>+</sup> lin<sup>-</sup> cells. Since CD71 and CD38 are more highly expressed by BM CD34<sup>+</sup> HPC than PB<sup>m</sup> CD34<sup>+</sup> HPC, this observation supports the proposition that differences in VLA-5 expression between sessile and circulating populations are at least linked to generic changes in the activation status of the study population.

LFA-3 is a counter-receptor for T-lymphocyte expressed LFA-2 and is more likely to play a role in HPC than in primary anchorage. VLA-5 mediates binding to fibronectin, an important constituent of the extracellular matrix of bone marrow stroma. It was therefore considered necessary to develop an assay with which to examine the potential functional role of CAM in general, and VLA-5 in particular, in HPC-stromal adhesion.

## **CHAPTER 4. DEVELOPMENT OF A <sup>51</sup>CHROMIUM ADHESION ASSAY TO STUDY FUNCTIONAL ADHESION OF HAEMATOPOIETIC CELL LINES.**

### **4.1 Introduction. p 177**

### **4.2 Optimisation of <sup>51</sup>Chromium Radiolabelling. p 180**

- .1 Cell radiolabelling. p 180.*
- .2 Definition of the resolution limits of the gamma counter. p 181.*
- .3 Definition of the resolution limits of cell numbers. p 181.*
- .4 Reduction of supernatant contamination. p 183.*
- .5 Estimation of the rates of cell toxicity and <sup>51</sup>Chromium elution. p 186.*
- .6 Correlation of <sup>51</sup>Chromium-labelling with actual cell numbers. p 186.*

### **4.3 Optimal Coating of the Carrier with Substrate. p 189**

- .1 KG1a incubation with substrate. p 189.*
- .2 Coating of tissue culture wells with fibronectin. p 191.*
- .3 Effects of the assay medium on carrier and substrate adhesion. p 192.*

### **4.4 Standardisation of the Adhesion Assay. p 196**

- .1 Preincubation with denatured BSA, washing and shaking. p 196.*
- .2 Investigation of the temporal kinetics and temperature dependence of the assay system. p 197.*
- .3 Dispersion and morphology of cells on the well surface. p 201.*
- .4 Concentration dependence of the assay. p 204.*
- .5 Replating efficiency of the non-adherent population. p 206.*
- .6 Reproducibility and quality control. p 207.*

### **4.5 Discussion and Formulation of Results. p 210**



#### 4.1 Introduction.

Although immunocytometry provides valuable information about the pattern of HPC adhesion molecule expression, it does not provide information on the functional status of those molecules, nor on the role various cell adhesion molecule - ligand systems play in the processes of endothelial homing and stromal engraftment. In order to explore these issues, it was necessary to develop an *in vitro* assay with which to study cellular adhesion to a range of substrates. Several criteria were considered to be of importance in the design of the assay: firstly, it should allow study of cell adhesion to both cellular and non-cellular substrates; secondly, it should allow correlation of adhesive behaviour with cellular phenotype - especially cell adhesion molecule expression; thirdly, both adherent and non-adherent populations should be assayed in order to reduce sampling errors; fourthly, relatively large numbers of cells should be enumerated, in order to improve the statistical power of the assay; finally, parameters should be chosen which would allow the assay to focus on the affinity of a given cell-substrate interaction (*i.e.* other variable factors such as the temporal kinetics of the interaction, or the maximal binding capacity of a substrate would be controlled at optimal levels).

Several approaches were considered. Direct visualisation of adherent cells using light / phase or fluorescence microscopy is fairly straightforward, but small numbers of cells must be used (1-200/well) or a selected microscopic field enumerated for larger cell numbers. The variation in the number of cells between wells, or between similar sized areas in the same well, is irregular even in a perfectly mixed sample, and conforms to a Poisson distribution [Dacie and Lewis, 1975]. The standard deviation (SD) of the distribution of cell numbers has been shown to be equal to  $\sqrt{m}$ , where  $m$  is the mean of the number of cells counted. Thus the inherent coefficient of variation (cv) of counts on 100 cells is 10%, on 200 cells is 7.1%, and on 1,000 cells is 3.2%. The situation is compounded by technical and observer variation. It is necessary to introduce  $10^4$  to  $10^5$  cells per well in order to achieve an acceptable cv, and it is clearly not possible to enumerate these by eye.

Semisolid clonogenic assays have been used by several groups to study HPC

adhesion. Gordon, Clark, Atkinson *et al.* [1990] used the CFU-blast assay and Liesveld, Abboud, Duerst *et al* [1989] the CFU-GM assay as their primary read-out. This kind of assay yields information on adhesive behaviour of a functionally defined subpopulation of HPC, and may be applied to cell lines. There are three important limitations which follow on from those discussed in Chapters 2.1 and 2.9.1: first, the phenotypic identity of the cell giving rise to a particular colony is unknown, making it difficult to directly correlate the behaviour of the adhesive cells with their cell adhesion molecule expression; secondly, the intrinsic dependence of clonogenic assays on the culture environment leads to problems of assessing the potential effect of the substrate on the cloning efficiency (leading to concern about the comparability of adherent and non-adherent HPC counts in the adhesion assay); thirdly, 1-200 colonies per assay are required to avoid problems with statistical accuracy and observer error similar to those encountered in the direct visualisation methods discussed above [Blackett, 1974].

Vital stains can be used to quantitate adherent cell populations, these include methylene blue [Rahilly & Fleming, 1993], or 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [Mosmann, 1983], crystal violet [Wilkins, Stupack, Stewart *et al*, 1991] and rose bengal [Gamble & Vadas, 1988]. These are not easily applicable to the study of cell adhesion to cellular substrates, because both test and substrate cells are stained by the detector system.

A radiolabelling approach was considered because it promised accurate and sensitive quantitation of large cell numbers against both cellular and non-cellular substrates, allowed cell phenotype and functional adhesion to be related, and was potentially applicable to enriched HPC populations and haematopoietic cell lines. The radiolabel should have an appropriate energy and half life and should remain cell-associated for the duration of the experiment, without impairment of cellular function. Chromium-51 ( $^{51}\text{Cr}$ ), technetium 99m and indium 111 or 113m are gamma-emitters, and all can be used for cell labelling.  $^{51}\text{Cr}$  produces a high energy, low abundance gamma-ray, and has a longer half life than the other elements (27.7d). On cellular uptake it is reduced from the hexavalent to the trivalent form,



and binds to intracellular proteins [Danpure & Osman, 1990]. The specific activity of an element is the radioactivity per unit mass. One becquerel is defined as one disintegration / second, and 1 kilobecquerel (kBq) is equivalent to 27 nanocuries (nCi) ( $37\text{ kBq}=1\mu\text{Ci}$ ).  $^{51}\text{Cr}$  sodium chromate was available with a specific activity of 13-22 GBq/mg at a reference date. The problem of identifying a specific weight and activity of sodium chromate was circumvented by using a fixed radioactivity for cell incubation. The sodium chromate was supplied as a stock solution by the radiopharmacy at the Royal Infirmary of Edinburgh at 2 weekly intervals, such that the radioactivity after one week (mid-point) was predicted to be  $200\text{ kBq}/80\mu\text{l}$ . Some degree of variability in the labelling index of the cells (see below) therefore arose from the age of the salt.

Critical aspects of the assay were considered to be labelling intensity (which would determine sensitivity) and uniformity, potential for functional alteration in cell adhesion, cell toxicity, leakage of  $^{51}\text{Cr}$  label into the supernatant (with potential redistribution to cellular substrate) and the reproducibility of the assay.

It was decided to develop the  $^{51}\text{Cr}$  adhesion assay using human haematopoietic cell lines in the first instance. These provided the practical advantages of ready availability of large numbers of cells, consistency and homogeneity of the study population within which lineage differentiation and adhesion molecule expression could be relatively closely defined. This strategy avoided confronting the problems associated with enrichment, heterogeneity and characterisation of HPC populations during establishment of the assay system, issues which could be returned to at a later date.

Adhesion of the leukaemic cell line KG1a to plasma fibronectin (pFn) and denatured bovine serum albumin (dBSA) was used as a test system to optimise the chromium labelling (Section 4.2), assess substrate preparation (Section 4.3) and standardise the adhesion assay (Section 4.4). The most informative way of analysing the results is discussed in Section 4.5. Later the assay was extended to the study of other purified extracellular matrix components (Chapter 5) and to *in vitro* stromal cultures (Chapter 6).

Storage, handling and disposal of radioactive materials was carried out in accordance with national and institutional guidelines, and was overseen by the local radiation protection supervisor.

## 4.2 Optimisation of $^{51}\text{Cr}$ Chromium Radiolabelling.

### 4.2.1 Cell radiolabelling.

$^{51}\text{Cr}$  radiolabelling was carried out a modification of the technique described by Gallin, Clark & Kimball [1972] and Butcher & Ford [1986].  $1 \times 10^7$  KG1a were removed from culture, washed in FCS, and cell viability checked by trypan blue dye exclusion. The cells were incubated with  $^{51}\text{Cr}$  in  $100\mu\text{l}$  PBS + 20% FCS for 1 hour at  $37^\circ\text{C}$  in an incubator with gentle occasional mixing by hand. Hardy & Minguell [1992] demonstrated that optimal  $^{51}\text{Cr}$  binding occurs when serum is a component of both the radiolabelling medium and the assay medium. The cells were pelleted by centrifugation at 200g for 5 min, and the supernatant removed. The cells were washed twice in 2ml culture medium, with removal of the supernatant following centrifugation each time. The supernatant and washes were pooled (*total discard count*). The cells were resuspended in the adhesion medium, which for most purposes was IMDM + 10% FCS (*i.e.* standard tissue culture medium) The cells were recounted and viability checked. The concentration was adjusted to  $1 \times 10^6/\text{ml}$ . 10% of the cells + medium were removed (10% of the *total cell count*), and the remaining cells were pelleted and 10% of the supernatant removed (10% of the *total supernatant count*).

The following parameters were defined:

*global count*: total cell count + total discard count.

$^{51}\text{Cr}$  uptake: total cell count / global count (%).

*labelling index (LI)*: total cell count /  $10^3$  cells.

*supernatant contamination*: total supernatant count / total cell count (%).

#### *4.2.2 Definition of the resolution limits of the gamma counter.*

The gamma counter was set up to detect gamma irradiation in the spectrum 240-400, and the peak  $^{51}\text{Cr}$  gamma emission was adjusted to fall within the centre of that range, as recommended by the manufacturer. 200kBq gave a count in the order of 800,000cpm, suggesting a detection efficiency of 6.7%, and a comparable count was obtained on a second gamma counter. The accuracy of the gamma counter improves with the total number of events counted, and is therefore directly related to the radioactivity of the sample, and the time spent counting. In practice, 15min was the maximum tolerable time per sample, if a single experiment was to be analysed over 24hrs. A radioactive decay of approximately 2% may be expected over a period of 24 hours, and therefore paired samples were analysed sequentially (*i.e.* the supernatant and adherent counts from each experiment and control grouped together), as averse to batched (all the supernatants counted first, followed by all the adherent layers).

An experiment was carried out to determine the variability of the count under these circumstances. Serial dilutions of  $^{51}\text{Cr}$  were made up and counted six times in succession (Table 4.1). The mean and standard deviation of serial counts on the same sample, and the coefficient of variation (cv) were calculated.  $^{51}\text{Cr}$  undergoes exponential decay with a half life of 27.7 days, predicting a deterioration of approximately 0.3% over the expected period of the experiment (4hrs). The absolute floor of detection is determined by background radiation (40cpm). The relative floor depends on the cv one is prepared to accept. A cv of less than 10% is probably acceptable for accurate quantitation, suggesting that this is not an important limitation (Table 4.1).

#### *4.2.3 Definition of the resolution limits of cell numbers.*

The floor of detection in terms of cells numbers is dependent upon the maximal achievable labelling index, which is itself dependent on the sodium chromate uptake by the cells, and on the specific activity of the chromate salt. Unfortunately sodium chromate is toxic, leading to cell death and leakage into the supernatant. The  $^{51}\text{Cr}$

**Table 4.1 Resolution Limits of the Gamma Counter using Serial Dilutions of <sup>51</sup>Chromium.**

<i>target cpm</i>	<i>measured cpm</i>	<i>coefficient of variation</i>
10 <sup>5</sup>	101,392 ± 433	0.43%
10 <sup>4</sup>	10,199 ± 71	0.70%
10 <sup>3</sup>	1,076 ± 9	0.83%
500	533 ± 6	1.13%
250	228 ± 4	1.86%
100	101± 2	2.31%
50	46 ± 1	3.20%
Blank	42 ± 2	5.14%

*Legend.* Serial dilutions of <sup>51</sup>Cr were made up and counted 6 times in succession. Result are quoted as mean ± standard deviation of 6 sequential analyses of the same sample. *Abbreviations.* *cpm*: counts per minute. *cv*: coefficient of variation. *target cpm*: the estimated number of counts that would be expected from the dilutions made. The gamma counter is capable of detecting radioactivity just above background with an acceptable degree of accuracy.

incubation is therefore a trade-off between adequate labelling and cell toxicity. Cell death and  $^{51}\text{Cr}$  elution manifest as elevated supernatant radioactivity, falsely allocated to the non-adherent fraction.

Several concentrations of  $^{51}\text{Cr}$  were compared for their effect on cell labelling (Table 4.2), number and viability: 100kBq/100 $\mu\text{l}$ , 200kBq/100 $\mu\text{l}$ , 400kBq/200 $\mu\text{l}$ , and a control incubation in FCS only. 20-60% FCS was added to adjust the concentration, and to maintain cell viability. Cell recovery and viability was similar in all 3 concentrations and control, suggesting that cell loss was predominantly due to loss of low buoyant density cells and debris during the washing / centrifugation steps, rather than cell toxicity. An adequate labelling index and acceptable supernatant contamination was achieved at 200kBq/ $10^7$  cells/100 $\mu\text{l}$ . Although the total chromium dose was less than that used by other groups, the sodium chromate and cell concentrations were at the upper limits of the range 400-2,000kBq /  $10^7$ - $10^8$  cells/ml (10 $\mu\text{Ci}$  /  $10^7$ - $10^8$  cells/ml = LI:2cpm /  $10^3$  human lymphocytes [Butcher & Ford, 1986]; 20 $\mu\text{Ci}$  /  $2 \times 10^7$  cells/ml [Gallin, Clark & Kimball, 1972]; 50 $\mu\text{Ci}$  /  $2$ - $4 \times 10^7$  cells/ml = LI:300cpm/ $10^3$  murine cell line [Hardy & Minguell, 1993]). Butcher comments that although doses as high as 100-200 $\mu\text{Ci}$  (3,700-7,400kBq) can be used, these lead to significant radiocytotoxicity [Butcher & Ford, 1986].

With a LI of 40cpm/ $10^3$  cells, an adhesion assay using  $10^5$  cells per well will resolve cell counts in the middle of the range with a cv of approximately 1%, and detect a floor of approximately 1% of the population with a cv of between 2 and 3% (Table 4.1).

#### *4.2.4 Reduction of supernatant contamination.*

The optimal number of washes post-incubation was assessed by serial centrifugation / washing steps in a single experiment (Table 4.3). Removal of the supernatant and 2 subsequent washes were sufficient to remove most discard  $^{51}\text{Cr}$  without subjecting the cells to excessive time or trauma. In subsequent experiments it was found that larger volume washes (approximately 2ml) allowed a



**Table 4.2 Effect of  $^{51}\text{Cr}$  Chromium Concentration on Labelling Index and Cell Viability.**

<i>Parameters</i>	<i>400kBq/200<math>\mu</math>l</i>	<i>200kBq/200<math>\mu</math>l</i>	<i>100kBq/100<math>\mu</math>l</i>
Total count*	1,284 $\pm$ 124	700 $\pm$ 68	208 $\pm$ 97
$^{51}\text{Cr}$ uptake	8.6 $\pm$ 4.4%	35.0 $\pm$ 7.0%	55.7 $\pm$ 7.2%
Labelling Index (LI) <sup>s</sup>	23.0 $\pm$ 6.8	44.0 $\pm$ 13.4	12.0 $\pm$ 7.3
Supernatant contamination	4.4 $\pm$ 1.2%	2.5 $\pm$ 2.8%	1.9 $\pm$ 0.7%
cell recovery <sup>@</sup>	51.1 $\pm$ 14.6%	56.4 $\pm$ 4.1%	47.8 $\pm$ 17.1%
cell viability <sup>@</sup>	>99%	>99%	>99%

*Legend.* Various concentrations of  $^{51}\text{Cr}$  compared for their effect on cell labelling. Results quoted as mean  $\pm$  standard deviation of 3 experiments. Total count\* quoted as thousands of counts per minute. Labelling index (LI)<sup>s</sup> defined as counts per minute /  $10^3$  cells. Cell recovery<sup>@</sup> counted by Neubauer chamber, and viability assessed by trypan blue dye exclusion. Parallel samples subjected to washing only showed a cell recovery of  $59.0 \pm 9.5\%$  and viability >99%. Satisfactory labelling and supernatant contamination was achieved at a concentration of 200kBq/200 $\mu$ l.



**Table 4.3 Effect of Serial Washing on the Supernatant Contamination.**

cell count	70,032
discard	365,572
wash 1	35,849 (51%)*
wash 2	9,263 (12%)
wash 3	10,172 (13%)
wash 4	10,167 (13%)
wash 5	5,555 (7%)
wash 6	3,073 (4%)
wash 7	1,952 (3%)
wash 8	2,469 (3%)

*Legend.*  $1 \times 10^7$  KG1a were incubated with 200kBq  $^{51}\text{Cr}$  in 100 $\mu\text{l}$  for 1hr for cell labelling. Serial washes were carried out and the residual supernatant contamination calculated at each step. Two washes were felt to be sufficient to remove most contamination. During subsequent studies it was found that increasing the wash volume to 2-4mls reduced contamination after two washes to approximately 2%.

reduction in the supernatant contamination to approximately 2% with two washes only.

#### *4.2.5 Estimation of rates of cell toxicity and $^{51}\text{Cr}$ chromium elution.*

A further study was carried out to assess the likely decline in cell numbers and viability, and resulting increase in supernatant contamination over the experimental period (4hrs).  $1 \times 10^7$  KG1a were labelled with 200kBq of  $^{51}\text{Cr}$  using the standardised technique. Five aliquots were made, and cells / supernatant separated by centrifugation at time 0, and at hourly intervals over 4hrs. Though cell numbers and viability remained remarkably constant, an approximate doubling of supernatant contamination was observed over this period, presumably due to leakage of  $^{51}\text{Cr}$  from the cells (Table 4.4). Where possible, the supernatant contamination was assayed on a 10% aliquot set aside and separated at the end of the experimental period. In practice, the supernatant contamination was rarely greater than 1-2% and never greater than 5%.

#### *4.2.6 Correlation of $^{51}\text{Cr}$ chromium-labelling with actual cell numbers.*

A series of experiments was carried out to determine how well actual cell numbers correlated with cell number estimated by  $^{51}\text{Cr}$ -labelling. KG1a were labelled using standardised technique, and a target range of concentrations was established by serial dilution. The volume of each assay was normalised to 500 $\mu\text{l}$ , and the actual cell number counted by Neubauer haemocytometer. The radioactivity of the assays was counted by gamma counter, and a predicted cell number was calculated based on the assay count and the LI for the experiment. Four experiments were carried out, and the results are tabulated (Table 4.5). A two-tailed paired  $t$  test showed no significant difference between actual and estimated cell numbers through the range  $2.5 \times 10^3$  to  $1 \times 10^6$  cells. At  $1 \times 10^3$  the predicted count was significantly greater than the actual count. This is not surprising because blank count for these four experiments was  $39 \pm 2\text{cpm}$ , and blank and cellular counts are additive. Therefore if, for example, the blank count is subtracted from the measured count for

**Table 4.4 Alterations in Cell Viability and Supernatant Contamination over the Expected Experimental Period.**

<i>time</i>	<i>number of cells</i>	<i>viability</i>	<i>cell count</i>	<i>supernatant count*</i>	<i>total count</i>
0hrs	2.3x10 <sup>6</sup>	>99%	172,767	2,753 (1.6%)	175,520
1hr	2.7x10 <sup>6</sup>	>99%	175,623	4,823 (2.7%)	180,466
2hrs	2.3x10 <sup>6</sup>	>99%	170,187	4,250 (2.4%)	174,437
3hrs	2.3x10 <sup>6</sup>	>99%	157,013	4,780 (2.9%)	161,793
4hrs	2.3x10 <sup>6</sup>	>99%	166,689	5,817 (3.4%)	172,504

*Legend.* There was a slight diminution of the cell counts over 4hrs. Supernatant counts expressed as a percentage of the cell count\*, showed a progressive increase (correlation coefficient  $r=+0.90$ , regression analysis  $y = 1.46 + 0.38. x$ ).

**Table 4.5 Correlation of <sup>51</sup>Chromium-Labelling with Actual Cell Numbers.**

<i>Target cell number</i>	<i>Actual cell number</i>	<i><sup>51</sup>Cr count</i>	<i>Predicted cell number</i>	<i>Significance (n)</i>
1x10 <sup>6</sup>	86 ± 16x10 <sup>4</sup>	18,635 ± 7,442	80 ± 16x10 <sup>4</sup>	ns (3)
5x10 <sup>5</sup>	46 ± 12x10 <sup>4</sup>	11,393 ± 5,738	39 ± 7x10 <sup>4</sup>	ns (4)
1x10 <sup>5</sup>	8 ± 2x10 <sup>4</sup>	1,980 ± 800	7 ± 1x10 <sup>4</sup>	ns (4)
5x10 <sup>4</sup>	4 ± 1x10 <sup>4</sup>	1,030 ± 659	4 ± 1x10 <sup>4</sup>	ns (4)
1x10 <sup>4</sup>	1 ± 0.25x10 <sup>4</sup>	299 ± 199	1 ± 0.6x10 <sup>4</sup>	ns (4)
5x10 <sup>3</sup>	5 ± 1x10 <sup>3</sup>	117 ± 17	7 ± 3x10 <sup>3</sup>	ns (3)
2.5x10 <sup>3</sup>	2.7 ± 0.6x10 <sup>3</sup>	87 ± 13	4.6 ± 2.0x10 <sup>3</sup>	ns (5)
1x10 <sup>3</sup>	0.6 ± 0.4x10 <sup>3</sup>	58 ± 8	2.6 ± 1.6x10 <sup>3</sup>	P<0.05 (4)

*Legend.* Results are quoted as the mean ± standard deviation of (n) experiments. A paired *t* test showed no significant differences between actual and estimated cell numbers (labelling index 27 ± 10 cpm/10<sup>3</sup> cells). <sup>51</sup>Cr count is an accurate reflection of cell numbers throughout the range 5x10<sup>3</sup> to 1x10<sup>6</sup> cells. Below this, background radiation makes a significant contribution to the <sup>51</sup>Cr count, giving rise to an overestimate of cell numbers.

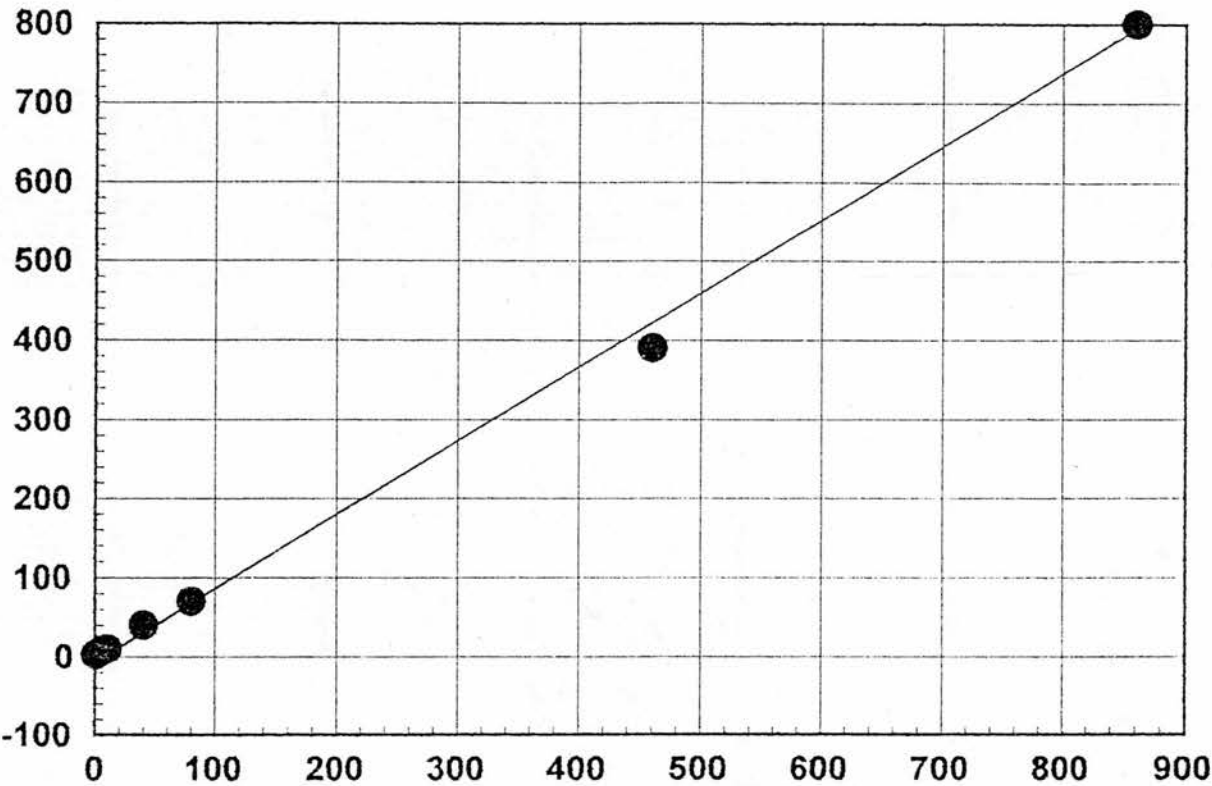
the  $1 \times 10^3$  series of assays, the corrected  $^{51}\text{Cr}$  count is  $19 \pm 7\text{cpm}$  and the corrected predicted cell count is  $1.1 \pm 0.4 \times 10^3$ , which is not significantly different from the actual count ( $p=\text{ns}$ ). The correlation coefficient calculated for mean data  $r = +1.00$  ( $n=8$ ), with a regression line described by the formula  $y = -0.36 + 0.91 \cdot x$  where  $x$  represents actual cell numbers and  $y$  represents predicted cell numbers (Figure 4.1). It was concluded, therefore, that  $^{51}\text{Cr}$ -labelling is an accurate representation of actual cell numbers over the range studied, though clearly accuracy does begin to degrade as the cell count approaches the absolute limit of detection at  $10^3$ .

### 4.3 Optimal Coating of the Carrier with Substrate.

#### 4.3.1 KG1a incubation with substrate.

The adhesion assay was standardised using  $^{51}\text{Cr}$ -labelled KG1a, and pFn and dBSA-coated 12 ( $4\text{cm}^2$ ) or 24 well ( $2\text{cm}^2$ ) tissue culture clusters. Triplicate wells within each tissue culture plate were incubated with 200 or  $100\mu\text{l}$  (respectively) of PBS containing  $0.05\text{mg/ml}$  of pFN, or 1% BSA denatured by boiling for 5min. These were allowed to dry completely at  $37^\circ\text{C}$  over 1hr. The coating concentration of pFN was therefore  $2.5\mu\text{g}/\text{cm}^2$ , consistent with that recommended by other groups [Giancotti, Comoglio & Tarone, 1986].  $2 \cdot 4 \times 10^5$   $^{51}\text{Cr}$ -labelled KG1a were added to each well, and the volume of assay medium was adjusted to 200- $400\mu\text{l}$ . This gave a working cell concentration of  $1 \times 10^5/100\mu\text{l}/\text{cm}^2$ . Cells were incubated with the substrate for 2hrs at  $37^\circ\text{C}$ , and the supernatant was removed by pipette. The wells were washed twice with assay medium with gentle agitation of the plate by hand to remove loose and non-adherent cells. The supernatant and washes were pooled (*supernatant count*) in a scintillation tube. The adherent cells were incubated for 15min with 0.1% Non-Idet in distilled water to lyse adherent cells, and removed by pipette (*adherent count*). The supernatant and adherent counts were measured by gamma counter as previously described. The total count (supernatant + adherent count) and % adherence ( $\text{adherent count} \times 100 / \text{total count}$ ) were calculated.

Figure 4.1 Regression Analysis Relating Actual and Predicted Cell Numbers Over the Target Range  $10^3$  to  $10^6$  Cells.



*Legend.* Regression analysis relating actual to predicted cell numbers over the working range  $10^3$  to  $10^6$  cells. *Abscissa:* actual cell number  $\times 10^3$ . *Ordinate:* Predicted cell number  $\times 10^3$ . Solid data points are observed values. The correlation coefficient  $r = +1.00$  with a regression line described by the formula  $y = -0.36 + 0.91.x$ . The full range of data is documented in Table 4.5.



#### 4.3.2 Coating of tissue culture wells with fibronectin.

A series of experiments was carried out to determine the conditions under which optimal discrimination between specific (fibronectin) and control (albumin) adhesion could be achieved.

An important initial consideration was the nature and characteristics of the carrier. Most plastics will adsorb protein from solution, though the avidity and capacity with which they do so may vary considerably. Coating of a plastic in excess of its capacity may lead to lower avidity binding of the protein to itself, with resultant instability or desorption of bound protein (prozone effect). In addition, the integrity of the protein may be compromised by binding, or the orientation / presentation may be inadequate, leading to reduced functional activity. It has been shown, for example, that when the surface chemistry of glass is modified by derivatization with alkyl chains containing end-groups of different polarities, there is little variation in the amount of fibronectin which adsorbs to the carrier, but dramatic variation in the adhesive behaviour of cells, presumably due to differences in protein conformation [Lewandowska, Balachander, Sukenik *et al.* 1989]. Finally, the carrier itself may bind cells, leading to difficulty in discrimination of specific binding against background. Twelve and 24 well polystyrene plates were used which had been prepared by the company by an electrical charging technique termed Corona discharge. This breaks the structure of the styrene ring and allows hydrophobic and ionic interaction with macromolecules and cells [personal communication: Costar UK Ltd.].

A study was carried out to assess optimal coating of the surface of the tissue culture wells. Parallel sets of triplicate wells in 2 separate polystyrene tissue culture plates were incubated with 100µl of 50µg/ml pFn in PBS, or 100µl of 1% dBSA in PBS. One plate was allowed to dry at 37°C for 1hr, whilst the other was simply incubated on the bench at ambient temperature. Both plates were then washed twice with 500µl IMDM 10% FCS per well, and the adhesion of  $1 \times 10^5$  KG1a / well studied in the manner described below. A paired *t* test carried out on the results of three replicate experiments, showed no significant difference between KG1a

adhesion to pFn under the two different coating conditions described, or similarly to dBSA . This suggests that the pFn is adsorbed onto the surface of the plastic from solution and maintained in a functional configuration (Table 4.6).

A group of experiments were carried out to assess optimal coating concentration of pFn, using the surrogate indicator of percentage KG1a adherence. Wells were incubated with 100µl of pFn in distilled water at a range of concentrations between 1 and 500µg/ml. It was confirmed that KG1a adherence rose to a plateau at 50µg/ml, corresponding to a maximal adsorption of between 0.5 and 2.5µg pFn / cm<sup>2</sup> (Table 4.7). This is comparable with the data of Giancotti, Comoglio & Tarone [1986], who showed that binding of murine cell lines to pFn *in vitro* achieved plateau at a fibronectin concentration of 25µg/ml, corresponding to adsorption of 0.5µg/cm<sup>2</sup> of the protein.

Experiments looking at KG1a adhesion to the plastic carrier (Section 4.3.3 and Table 4.8 *vide infra*), demonstrated very substantial background binding, which was markedly reduced by a coating step (with dBSA), and also by the presence of FCS in the assay medium. The presence of a surfeit of denatured protein is clearly a prerequisite for the discrimination of specific from background binding, though there is a risk that serum proteins may compete with the purified substrate protein for space on the carrier.

#### 4.3.3. *Effects of the assay medium on carrier and substrate adhesion.*

The influence of the assay medium on substrate adhesion was examined. KG1a were <sup>51</sup>Cr labelled using standard technique, and thereafter washed and handled in IMDM, IMDM + 10% FCS, or HBSS containing 10mM (0.4g/l) EDTA. The effects of these assay media on KG1a adhesion to tissue culture plastic, pFn and denatured BSA, was studied, and the results are summarised in Table 4.8. KG1a adhesion was significantly greater to pFn than to dBSA in IMDM (p<0.01) and IMDM + 10% FCS (p<0.01), but not in HBSS + EDTA. There was no significant difference in binding to pFn using IMDM or IMDM + 10% FCS as the assay medium, suggesting that FCS was not competing with the pFn for carrier space - at least at this

**Table 4.6 Effects of Coating Conditions on KG1a Adherence to Fibronectin and Albumin Coated Tissue Culture Wells.**

<i>substrate</i>	<i>wet coating</i>	<i>dry coating</i>	<i>significance</i>
fibronectin	71.7± 8.6% (3)	65.5± 8.4% (3)	ns
albumin	29.4±15.3% (3)	28.9± 5.3% (3)	ns
tissue culture plastic		77.6± 2.9% (3)	

*Legend.* Results are quoted as mean % adherence ± standard deviation (number of experiments). Statistical significance of the difference between wet and dry coating conditions was determined by paired Student's *t* test. Adherence to naked tissue culture plastic and fibronectin-coated wells was significantly greater than that to albumin-coated wells ( $p<0.001$  and  $p<0.01$  respectively), but there was no significant difference between plastic and fibronectin adherence, indicating the need for a blocking agent to enable discrimination of specific binding against background.

**Table 4.7 Binding of KG1a to Fibronectin under Varying Coating Concentrations.**

<i>coating concentration</i>	<i>KG1a adherence (n)</i>
1 µg/ml	16.8 ± 12.9% (4)
5 µg/ml	24.3 ± 12.9% (4)
10 µg/ml	33.5 ± 15.7% (4)
50 µg/ml	49.4 ± 16.0% (4)
100 µg/ml	45.6 ± 16.3% (4)
500 µg/ml	49.1 ± 16.4% (4)

*Legend.* A group of experiments was carried out to assess optimal coating concentration with pFn using KG1a adherence as a surrogate indicator. Results are quoted as mean % adherence ± standard deviation (n = number of experiments). KG1a adherence rose to a plateau at 50µg/ml.

**Table 4.8 Effects of Assay Medium on KG1a Adhesion to Various Substrates.**

<i>substrate</i>	<i>IMDM</i>	<i>IMDM + 10% FCS</i>	<i>RPMI + 10% FCS</i>	<i>HBSS + EDTA*</i>
pFn	66.2 ± 15.9%	61.1 ± 5.9%	48.2 ± 6.0%	16.3 ± 12.2%
dBSA	15.9 ± 6.7%	10.1 ± 4.8%	15.2 ± 10.5%	20.9 ± 11.3%
plastic	69.1 ± 2.5%	13.2 ± 6.2%		

*Legend.* Result are quoted as mean % adherence ± standard deviation of 4 replicate experiments. The pFn and dBSA data is from a different set of experiments than the tissue plastic data. Paired *t* test was used to compare data from concomitant experiments. Non-paired *t* test was used to compare data from separate series of experiments in IMDM and RPMI. HBSS + EDTA\*: refer to Chapter 5.4.1.

-----

concentration. pFn binding in these media was superior to that in HBSS + EDTA ( $p<0.01$  and  $p<0.01$  respectively) suggesting that divalent cations are essential for pFn-specific binding (Chapter 5.4.1). There was no significant difference in binding to dBSA between the 3 assay media. Adherence to tissue culture grade plastic was substantial, and was markedly reduced in the presence of FCS ( $p<0.001$ ). Routine assays were therefore carried out in IMDM + 10% FCS (*assay medium*). At a later date it became more attractive on financial grounds to use RPMI as the base medium. KG1a binding to pFn in RPMI and RPMI + 10% FCS was significantly greater than that to dBSA ( $p<0.02$  and  $p<0.01$  respectively: paired *t* test), and showed no significant difference between the two media ( $p=ns$ : paired *t* test). Non paired *t* tests showed no significant difference in KG1a binding to pFn between IMDM and RPMI ( $p=ns$ ), though binding in IMDM + 10% FCS was slightly superior to that in RPMI + 10% FCS ( $p<0.05$ ). Again dBSA binding showed no

dependence on the type of medium. Some authors have claimed that FCS enhances cell adhesion through the presence of a serum-spreading factor (probably vitronectin) and other plasma glycoproteins such as fibronectin. This was not confirmed in these experiments, and there may be several explanations for the discrepancy: that FCS does vary considerably from batch to batch, that heat inactivation of the FCS denatures structural proteins or that haematopoietic cell lines may not adhere to the putative serum spreading factor (see Chapter 5.2).

Overall, the data for sections 4.3.2 and 4.3.3 support the validity of using Costar tissue culture plates as a satisfactory protein carrier for adhesion experiments, provided background cellular adhesion to the plastic is adequately blocked by the test protein and / or FCS in the assay medium.

#### **4.4 Standardisation of the Adhesion Assay.**

##### *4.4.1. Pre-incubation with denatured BSA, washing and shaking.*

The extent of specific adhesion of cells to substrate is dependent on whether complete blockade of background adhesion has been achieved, and the stringency of the washing and shaking steps. *i.e.* the distractive phase of the assay. Since these factors are compound in their effect on cell-substrate adherence in the experimental system, they were examined in tandem. Parallel triplicate wells were coated with pFn or dBSA. In one plate, each well was further incubated with 0.5ml of 1% dBSA in PBS for 15min, which was then removed by pipette just prior to introduction of  $^{51}\text{Cr}$ -labelled KG1a. Wells in the other plate were not subjected to this step. In each plate, non-adherent KG1a in parallel triplicate wells were removed with the supernatant, and the adherent KG1a then subjected to none, one or two washes with 200 $\mu\text{l}$  of assay medium. Washes were added by tipping the plates slightly, and adding the media down the side of the well from a 200 $\mu\text{l}$  pipette. The plates were swirled a few times by hand, and the supernatant / wash removed after tipping the plate in the opposite direction. This approach reduced the possibility of directly dislodging or aspirating adherent cells with the stream from the pipette. A total of 6 triplicate assays was therefore carried out, in each of 2 experiments.



As expected, both pFn and dBSA binding were highest when the supernatant was removed without washing, but the differential between dBSA and pFn binding improved with washing, both with and without dBSA preincubation. pFn adhesion was significantly impaired by dBSA preincubation after 2 washes ( $p < 0.001$ ), whilst this made no difference to background (dBSA) adhesion (Table 4.9).

In a separate experiment the effect of tipping the plate twice in both directions by hand ("shaking") before removing each wash was compared with not doing so (*i.e.* simply removing the supernatant). The results of this simple experiment are detailed in Table 4.10, and demonstrated that non-shaken cultures gave rise to significantly greater binding, and less discrimination between pFn and dBSA adhesion.

In summary of this composite data, it was found that removing the supernatant and washing the substrate twice, with gentle shaking of the plate by hand each time, gave the best discrimination between KG1a adhesion to pFn and dBSA. A dBSA pre-incubation step was considered unnecessary in an assay medium containing FCS, provided that adequate washing of loosely adherent cells was carried out.

#### *4.4.2 Investigation of the temporal kinetics and temperature-dependence of the assay system.*

A study was made of the temperature dependence of the assay, by incubating KG1a with pFN and BSA substrates at 4°C, ambient temperature (approximately 20°C), and at 37°C in separate tissue culture plates. The results are summarised in Table 4.11. Background adherence was not affected by temperature, but pFn binding was abrogated at 4°C. There were no significant differences between binding activity at ambient temperature and at 37°C.

A study of the temporal dependence of the incubation was also made (Table 4.12). Radiolabelled KG1a were incubated with pFN and BSA substrates for 0 min (added and then immediately removed), 15min, 30min, 1hr, 2hrs and 4hrs. Background adherence remained relatively constant, with a floor even following negligible incubation time (sample 0). Fibronectin-specific adhesion rose from this

**Table 4.9 Effects of Pre-Incubation with 1% Denatured BSA and Number of Washes on KG1a Adherence to Fibronectin and Albumin.**

<i>substrate</i>	<i>no wash</i>	<i>one wash</i>	<i>two washes</i>
<i>albumin pre-incubation</i>			
fibronectin	32.8 ± 6.2%	11.8 ± 1.6%	16.6 ± 0.1%*
albumin	8.5 ± 0.1%	2.0 ± 0.6%	1.2 ± 0.1%~
significance	p<0.05	p<0.02	p<0.001
<i>no albumin pre-incubation</i>			
fibronectin	41.8 ± 4.6%	34.1 ± 2.3%	21.2 ± 0.1%
albumin	16.4 ± 3.8%	3.4 ± 0%	1.1 ± 0.3%~
significance	p<0.05	p<0.01	p<0.001

*Legend.* Results are quoted as mean % adherence ± standard deviation (n=2). Significance levels are those of the difference between fibronectin and albumin adherence under the conditions specified (paired Student's *t* test). Following 2 washes, the difference between fibronectin adherence with and without an albumin pre-incubation step is highly significant (p<0.001)\*, whilst that between albumin adherence with and without an albumin pre-incubation step is not~.

**Table 4.10 Effects of "Shaking" or "Not-Shaking" the Plate between Washes.**

<i>substrate</i>	<i>shaken plates</i>	<i>non-shaken</i>
pFn	38.4 ± 11.8% (4)	63.4 ± 6.2% (4)
dBSA	8.9 ± 4.8% (3)	48.6 ± 16.7% (4)

*Legend.* Mean % adherence ± standard deviation of (n) replicate experiments. Paired *t* tests demonstrated pFn and dBSA adhesion to be significantly higher under non-shaken conditions compared to shaken (both  $p < 0.01$ ). Moreover, the difference between pFn and dBSA adhesion was significant in the shaken plates ( $p < 0.02$ ), but not in the non-shaken plates ( $p = ns$ ).

-----

**Table 4.11 Temperature Dependence of the Assay System.**

<i>Substrate</i>	<i>+4°C</i>	<i>AT</i>	<i>37°C</i>
pFn	23.0 ± 5.8%	52.3 ± 2.1%	51.0 ± 1.9%
dBSA	21.0 ± 4.2%	22.9 ± 3.6%	19.1 ± 0.5%

*Legend.* Results are presented as mean % adherence ± standard deviation of 4 replicate experiments. Background adherence was not affected by temperature, but pFn adherence was reduced at +4°C. There was no significant difference between adhesion at ambient tempertaure (AT) and 37°C.

**Table 4.12 Temporal Dependence of the Assay System.**

<i>substrate</i>	<i>0min</i>	<i>15min</i>	<i>30min</i>
pFn	2.6 ± 1.1%	16.9 ± 8.6%	21.5 ± 13.5%
dBSA	4.4 ± 2.1%	14.7 ± 8.7%	8.8 ± 6.2%

<i>substrate</i>	<i>1hr</i>	<i>2hrs</i>	<i>4hrs</i>
pFn	36.2 ± 20.3%	27.8 ± 34.6%	32.0 ± 22.8%
dBSA	6.5 ± 5.7%	4.8 ± 2.7%	5.1 ± 1.2%

*Legend.* Data is presented as the mean % adherence ± standard deviation of 4 replicate experiments. Background adherence remained relatively constant throughout, pFn adherence rose to a plateau at 1hr.

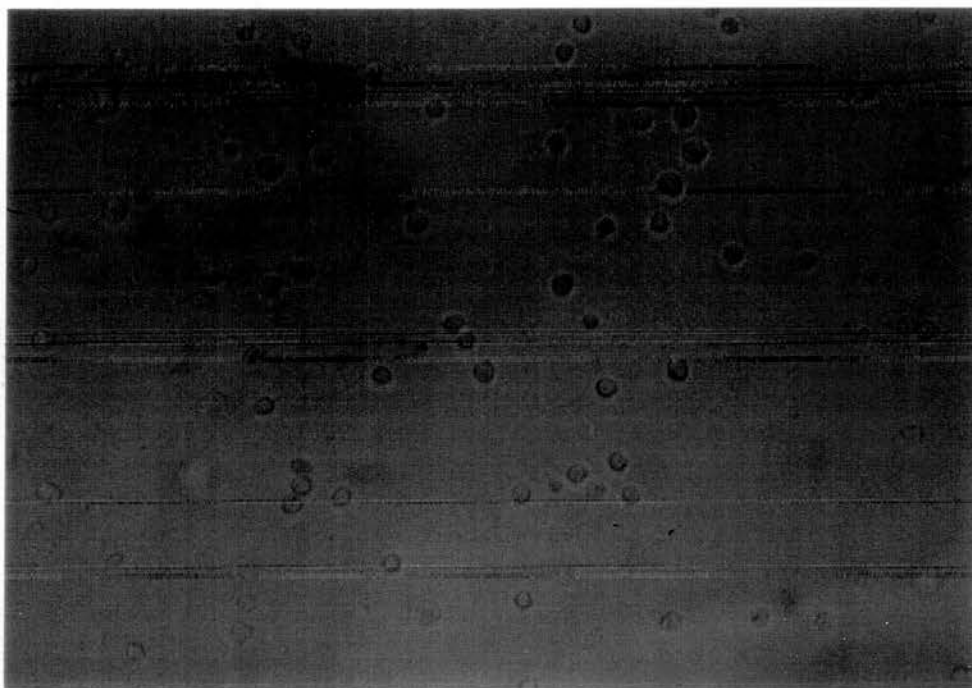
floor to a plateau following 1hr incubation (Table 4.12). The kinetics of attachment are similar to reported rates for other cell types [Jauregui, McMillan, Driscoll *et al.* 1986; Hardy & Minguell, 1993].

It seems unlikely that the assay will be useful in studying adhesive phenomena as they evolve because of the inherent delay in sedimentation of the leucocytes to the substrate surface. 400 $\mu$ l of medium occupies a depth of 2mm over a 2cm<sup>2</sup> surface area. Calculations based on a leucocyte sedimentation index (in plasma) of 13.6mm/hr (95% range 3.7 - 23.4mm/hr) [Diem & Lentner, 1970] suggest that the cells should settle to the adhesive surface within 8.8min (95% range 32.3 - 5.1min). In addition, there appears to be a residual floor of 2-5% of cells in the adherent fraction even after negligible incubation.

#### *4.4.3 Dispersion and morphology of cells on the well surface.*

The dispersion of the cells on the well surface was examined by inverted microscopy. In initial experiments it was found that cells tended to be concentrated around the perimeter of the circular well, despite thorough mixing by hand. A series of experiments revealed that the ratio of cells / high-power field in the periphery compared to the centre of the assay dish was between 4:1 and 7:1. It was found that increasing the volume of the suspension medium to 400 $\mu$ l eliminated this problem, giving rise to a uniform cell distribution. The reason for this phenomenon may be that the depth of suspension medium is less in the centre of the well than towards the periphery due to convexity of the centre of the well base or formation of a meniscus due to surface tension at the periphery, or may relate to the pattern of convection currents within the well. At an incubating volume of 400 $\mu$ l / 2cm<sup>2</sup> well, distribution of KG1a within the culture well was found to be uniform both during the adhesion phase, and following removal of the supernatant (Figure 4.2). An experiment carried out to determine whether the suspension volume made any difference to the %KG1a binding to pFn, failed to show any such difference (Table 4.13).

**Figure 4.2 Adhesion of KG1a to Plasma Fibronectin.**



*Legend.* Uniform dispersion of KG1a adherent to the surface of a pFn-coated well, 1hr following suspension in 400 $\mu$ l of assay medium. No change in the morphology of the adherent cells was apparent. (magnification x10).



**Table 4.13 Effects of Variation in Suspension Medium on KG1a Adhesion to Fibronectin.**

	<i>200µl medium</i>	<i>400µl medium</i>	<i>significance</i>
% adherence	58.3 ± 12.5%	48.2 ± 6.0%	ns

*Legend.* Data is presented as the mean % adherence ± standard deviation of 4 replicate experiments. There was no significant difference in the adherence of KG1a to pFn in the 2 volumes studied (paired *t* test).

-----

A second consideration pertained to the distribution of adherent cells in the wells following removal of the supernatant and non-adherent cells. Microscopic examination revealed that vigorous washing led to an uneven distribution due to dislodgement of some adherent cells. Careful removal and addition of fluid by pipette from the side of the tilted culture dish avoided the problem.

The question of whether the reproducibility of the assay could be improved by centrifugation of the plate at the beginning of the incubation was explored. Parallel experiments were carried out to compare KG1a adhesion to pFN and BSA, with and without centrifugation of the plate for 10min at 500g. The results showed no significant difference between adhesion to either substrate under these circumstances (Table 4.14), confirming that apposition of cells and substrate was not a limiting factor in the assay, and demonstrating that centrifugation was not effective in reducing inter-experimental variability. Microscopic examination of the plates revealed that non-centrifuged plates showed an even dispersion of cells over the test surface, whilst those which had been centrifuged showed concentration of cells at the periphery of the plate, with a predominance at the dependent side. Since a horizontal plate centrifuge was not available, this avenue was not explored further.

**Table 4.14 Effect of Centrifugation on Overall Cell Adhesion.**

<i>substrate</i>	<i>centrifugation</i>	<i>no centrifugation</i>
pFn	29.6 ± 16.0%	34.1 ± 22.7%
dBSA	19.6 ± 15.8%	22.8 ± 20.4%

*Legend.* Data is presented as the mean % adherence ± standard deviation of 3 replicate experiments. No significant difference could be demonstrated between adherence with or without centrifugation (non-paired *t* test).

---

#### *4.4.4 Concentration dependence of the assay.*

There are two relevant cell concentrations, the volumetric concentration and the two-dimensional surface concentration. The volumetric concentration is important because adhesion may be inhibited by cell-secreted proteins [Gjessing & Seglen, 1980]. The surface concentration is important because it reflects the degree to which cells may physically interact or compete for available sites at the adhesive surface. For leucocytes approximately 20µm in diameter, total surface coverage with spheres corresponds to  $2.5 \times 10^5$  cells / cm<sup>2</sup>.

The dependence of background and specific adherence on concentration was studied using standard technique, and 24 well pFn and dBSA coated tissue culture plates (surface area 2cm<sup>2</sup>). Adhesion of radiolabelled KG1a were studied at concentrations between  $1 \times 10^4$  and  $1 \times 10^7$ /well. No cell-cell aggregation was observed microscopically even at higher volumetric concentrations. The results of 3 experiments are summarised in Table 4.15. At low levels of KG1a ( $1-2 \times 10^4$ ), background albumin adherence was higher than expected. However, it should be recalled that the LI for these 3 experiments was  $36 \pm 7$  cpm /  $10^3$  cells set against a standard background of  $41 \pm 1$  cpm. The absolute floor is therefore 10% of  $1 \times 10^4$  cells and 5% of  $2 \times 10^4$  cells, but only 1% of  $1 \times 10^5$  cells. Apart from this anomaly,

**Table 4.15 Concentration Dependence of the Assay System.**

<i>cell (n)</i>	<i>pFn</i>	<i>dBSA</i>
1x10 <sup>4</sup>	41.2 ± 13.6% (4,120)	13.8 ± 2.4% (1,380)
2x10 <sup>4</sup>	42.3 ± 14.8% (8,460)	14.2 ± 1.9% (2,840)
1x10 <sup>5</sup>	38.9 ± 9.2% (38,000)	5.4 ± 2.8% (5,400)
2x10 <sup>5</sup>	36.0 ± 10.5% (72,000)	5.3 ± 3.9% (10,400)
1x10 <sup>6</sup>	41.2 ± 19.5% (412,000)	5.6 ± 1.7% (56,000)
2x10 <sup>6</sup>	43.2 ± 2.9% (864,000)	6.0 ± 1.4% (120,000)
1x10 <sup>7</sup>	8.6 ± 2.7% (860,000)	1.5 ± 0.8% (150,000)

*Legend.* *cell(n)* represents the number of <sup>51</sup>Cr-labelled KG1a added to each 2cm<sup>2</sup> well of a 24 well plate. Data is presented as the mean % adherence ± standard deviation of 3 replicate experiments, with the calculated absolute adherent cell numbers in parentheses.

percentage adherence remained remarkably constant over the range of  $1 \times 10^4$  to  $2 \times 10^6$  cells / well, for both pFn and BSA adherence. This implied that a fixed proportion of KG1a were capable of adherence to these substrates. At  $1 \times 10^7$  cells / well, the percentage adherence fell sharply, and when absolute adherent cell numbers were calculated, a ceiling of  $8.6 \times 10^5$  KG1a / pFn-coated well, and  $1.5 \times 10^5$  KG1a / BSA-coated well were defined. Total well surface coverage is calculated to be achieved at  $5 \times 10^5$  cells. These figures are highly dependent on the defined experimental conditions, but do suggest that a reproducible maximal binding capacity exists, though whether due to occupation of a finite number of binding sites, or steric hindrance at the highest density is not discriminated.

#### *4.4.5 Replating efficiency of the non-adherent population.*

A series of experiments was carried out to establish the extent to which non-adherent and loosely-adherent cells present in the supernatant were capable of binding to the test substrate in a second assay. Triplicate assays were established at a concentration of  $4 \times 10^5$  cells /  $2 \text{ cm}^2$  well, against substrates of pFn and dBSA, using the standardised protocol. The supernatant removed after 2hrs was immediately transferred to a second series of wells containing the same test substrate, and incubated for a second 2hrs. The supernatant and adherent fractions from the second phase were considered to equate to the supernatant from the first. Percentage adherences were established for both phases, and the results are shown in Table 4.16. Binding to pFn fell significantly on replating ( $p < 0.05$ , paired  $t$  test), whereas the fall in dBSA binding fell short of significance on the paired  $t$  test. There was no significant difference between pFn and dBSA adhesion during secondary adhesion, though this may reflect the high variability of the pFn adhesion. The lower proportion of dBSA-adherent cells following replating is difficult to rationalise. It is possible that background binding is partially dependent on physical characteristics of the cells such as size or ionic charge.

**Table 4.16 Effects of Replating Non-Adherent Cells.**

<i>substrate</i>	<i>primary adhesion</i>	<i>secondary adhesion</i>	<i>significance (paired t test)</i>
pFn	34 ± 4%	13 ± 9%	p<0.05
dBSA	7 ± 0.6%	4 ± 1%	ns

*Legend.* Results quoted as mean ± standard deviation of 3 experiments. pFn is significantly greater than dBSA during primary adhesion (p<0.01) but not during secondary adhesion (ns) (paired *t* test). Percentage adhesion to pFn fell significantly on replating whereas that to dBSA did not.

---

#### *4.4.6 Reproducibility and quality control.*

To achieve maximum reproducibility, each assay was carried out in triplicate within a single tissue culture plate, in tandem with triplicate internal positive and negative controls. In addition, the labelling parameters and control assays were monitored over the period of research to establish the mean and coefficient of variation of the experimental system, and to ensure that there was no drift in the controls due to, for example, change in operator technique. The stability of the labelling indices and control adhesion indices is documented in Table 4.17. The labelling indices were considered to be satisfactory, with no evidence of drift. A key problem was found to be the variability of dBSA as a negative control. A meta-analysis of 23 experiments carried out over a 6 month period showed considerable variation in percentage binding to pFn and to dBSA. An analysis of the *differences* between pFn and dBSA binding showed an overall 30.6% difference which was significant at p<0.001 (Table 4.18). Further analysis of this data with correction for the level of dBSA binding showed that the assay remained valid at all



**Table 4.17 Stability of the Labelling Indices.**

*Labelling indices*

global count	627 ± 151 (22)
<sup>51</sup> Cr uptake	0.40 ± 0.14 (22)
Labelling Index	34 ± 12 (21)
Supernatant contamination	2.8 ± 2.1% (19)

*adherence indices*

pFn	47.6 ± 19.7% <i>cv</i> 41.4 (23)
dBSA	17.1 ± 11.3% <i>cv</i> 66.1 (23)

*Legend.* Results quoted as mean ± standard deviation of *n* experiments. *cv*. coefficient of variation.

**Table 4.18 Subanalysis of Fibronectin and dBSA Adhesion.**

<i>dBSA adherence</i>	<i>mean difference ± SD pFn% - dBSA%</i>	<i>significance (n)</i>
global	30.6 ± 18.4%	p<0.001 (23)
<10%	33.2 ± 20.5%	p<0.01 (7)
10-20%	33.5 ± 18.9%	p<0.01 (8)
>20%	25.3 ± 17.2%	p<0.01 (8)

*Legend.* Mean difference between pFn and dBSA binding ± standard deviation is quoted for a series of experiments, and sub-analysed for experiments in which background (dBSA) binding was <10%, 10-20%, and >20%. Significance levels (paired *t* test) and number of experiments analysed (*n*) are documented.



**Table 4.19 Background Adhesion of KG1a to Different Batches of BSA.**

batch #1 dBSA	44.8 ± 25.9% (4)
batch #2 dBSA	47.2 ± 23.5%
BSA (not denatured)	43.8 ± 21.3%

*Legend.* Results quoted as mean ± standard deviation of 4 experiments. A paired *t* test failed to show any significant differences between these substrates.

-----

levels of background binding.

A series of experiments was carried out to determine whether control binding was dependent on the extent of denaturation or age of the dBSA. A low stringency wash was used so that differences in dBSA adhesion would be detectable if present. No batch effect could be demonstrated (Table 4.19).

#### **4.5 Discussion and Formulation of Results.**

The objective of the work discussed in this Chapter was to establish an assay system with which to study functional HPC adhesion. A standardised methodology was developed and optimised, and its reproducibility demonstrated. The potential strengths and limitations of the assay system were also explored.

<sup>51</sup>Cr was used to label human haematopoietic cell lines with minimal cell toxicity or chromium leakage. The labelling index is sufficient to resolve 1% of a population, provided a minimum of 10<sup>5</sup> cells are used per test. There is a theoretical concern that chromium could induce intracellular protein changes and lead to abnormal adherence within the test system. Hardy & Minguell (1993) have carried out parallel studies with <sup>51</sup>Cr and <sup>35</sup>S-methionine metabolic labelling on murine cell lines, and found no difference in their adhesion to a variety of substrates.

Costar tissue culture grade plastics proved to be a satisfactory carrier, providing adequate adsorption of protein from solution and maintaining functional orientation. Cellular adherence to tissue culture plastics is marked, but is effectively blocked by

coating with protein, and additionally by FCS in the assay medium. The presence of FCS does not affect pFn binding, and therefore permits differentiation of specific binding from background. An additional incubation step with dBSA appears unnecessary, and may compete with substrate for carrier binding, or inhibit specific cell binding.

The most important determinant of cellular adherence within the assay system is the technique used to remove the non-adherent and loosely adherent cells during the distractive phase of the experiment. Removal of the supernatant and 2 washes, with gentle mixing by hand, provided the greatest discrimination between substrate and background binding, but also proved the greatest source of inter-experimental variability. Percentage binding is highly dependent on the experimental conditions, especially stringency of washing, and should be seen as an indicative measure of the adhesive affinity of the test population as a whole for the test substrate. Replating experiments suggest that the primary non- / loosely-adherent population does have less binding affinity than the primary adherent population. The data derived from the concentration dependence of the assay suggests that a reproducible maximal binding capacity can be established for a given cell population and substrate under defined experimental conditions.

Taken overall, these data suggest the presence, in an apparently homogeneous population, of cells with varied but continuously distributed affinity for a given substrate, rather than the presence of two discrete populations (adhesive and non-adhesive). Cellular binding affinity is likely to depend on the density of expression and the activation state of one or more cell adhesion molecules. At the same time, an apparently homogeneous substrate is likely to display a distribution of binding domains which differ in affinity due to variation in accessibility and conformation. Percentage adherence and maximal binding capacity are therefore highly dependent on the technique used to distract the non-adherent population. Too little or too much washing stringency is likely to lead to loss of discriminatory power of the assay. Optimisation and standardisation of the distractive technique was therefore a key element in the development of the assay.

The question arose as to whether dBSA was the best "negative control" for these experiments. It became clear from studies on other extracellular matrix (ECM) components (Chapter 5.2) that the absolute floor of adhesion was less than that observed in the dBSA-coated wells. It is unclear whether this was due to a specific inhibitory feature of some ECM components (for example the negative charge associated with sulphated proteoglycans), an inherent (but non-specific) "stickiness" of denatured proteins or inadequate coverage of plastic. Given that different batches of dBSA showed little difference in background binding, it was thought that variability in the assay was primarily dependent on technical variation such as washing stringency, which reinforced the importance of the internal negative controls.

Three approaches to the formulation of the results were considered. The most comprehensive approach is to carry out each assay at a series of concentrations, in order to establish a maximal binding capacity (Section 4.4.4). To carry this out in triplicate within each experiment, with an internal negative control would require in the order of  $10^8$   $^{51}\text{Cr}$ -labelled cells per experiment. This was regarded as unfeasible for routine use given the time and resources available. Adhesion was assayed at a single concentration at  $1\text{--}2 \times 10^5$  cells /  $2\text{cm}^2$  representing 20-40% of total surface coverage, and allowing a single experiment to be completed with around  $10^6$  cells. The results from such an experiment can be quoted "as seen", with no attempt to correct for inter-experimental variability. Some groups have considered albumin adherence to be non-specific, and that the test substrate-specific adherence should be calculated by subtraction of albumin adherence from test substrate adherence. There are both theoretical and practical problems with this approach. The conceptual problem is similar to that discussed in the interpretation of dual immunofluorescence data, *i.e.* that since the character of the cells adherent to albumin is indeterminate, and may or may not be representative of the population as a whole, they may not be legitimately subtracted from the cells which adhere to the test substrate. Indeed the nomenclature of specific and non-specific adherence contains a mechanistic implication which is not warranted by the informative

content of the assay. For example, adherence to plastic is greater than that to albumin, but is not mediated by plastic-specific cell adhesion molecules. Conversely, adhesion to pFN can only be said to be "specific" if it can be demonstrated to be mediated by a unique adhesion pathway, which is not involved (for example) in adhesion to other ECM components. There are also practical difficulties with this approach where substrate adherence is less than that to dBSA (Chapter 5.2). Some groups have quoted results as a *binding index*, defined as the ratio of percentage substrate adherence (numerator) to percentage albumin adherence (denominator) [Hardy & Minguell, 1993]. This approach normalises the substrate adherence to that of the neutral background substrate, and goes some way towards reducing inter-experimental variability due to washing and shaking technique. The weakness of formularising results in this way is the somewhat arbitrary nature of the calculation, and rigidity in practical application. I elected to evaluate the statistical significance of results using a two-tailed paired *t* test to compare substrate binding with concurrent controls over 4 replicate experiments. Where binding results were compared with those from a different series of experiments, a two-tailed, non-paired *t* test was used. The use of these statistical tests is based on the assumption that variation in cell binding between similar experiments is a normally distributed continuous variable. Two-tailed tests are used because substrate binding may be higher or lower than control. This approach allowed flexibility in comparing substrate to control adherence, evaluating adherence to different substrates, and examining the effects of activation or blockade on specific interactions. However it should be noted that the criteria for a given *p* value in a paired *t* test are more stringent than those in a non-paired because of the lower number of degrees of freedom allowed when entering the table of the *t* distribution. The presentation of the analysis as a test with 2 possible outcomes (significant or non-significant) does over-simplify the interpretation of the results. The choice of a particular cut-off for statistical significance is somewhat arbitrary, and depends on the probability of a Type I error one is prepared to accept (the chance that an apparently "significant" result is, in fact, normal). In view of the

multiple comparisons made during some experiments (*e.g.* Chapter 5.2), and with the tacit support of convention, the term "statistical significance" was reserved for  $p$  values of  $<0.01$ . Adhesive interactions of biological significance may fail to achieve statistical significance due to inadequate power of the study, a reflection of small number of experiments and / or high inter-experimental variability. Stringent criteria for statistical significance increase the risk of a Type II error (*i.e.* that a biologically significant difference is missed) [Altman, 1994].

In summary, unless otherwise stated, results are quoted as the mean % adherence ( $m$ )  $\pm$  the standard deviation (SD) of several replicate experiments, which gives an indicative measure of the overall binding affinity between the cell population and the substrate under the defined experimental conditions. The result of a two-tailed paired or non-paired  $t$  test is quoted in parenthesis as a  $p$  value, and is used to compare test data with internal controls or with an external set of experiments respectively. The  $p$  value provides a measure of the probability of obtaining the observed result if there is (in fact) no difference in the adhesive behaviour of the two populations under comparison. Because of the somewhat arbitrary nature of the conventional cut-off,  $p$  values of  $>0.05$  are considered non-significant, and those  $<0.01$  are considered significant, and those between 0.01 and 0.05 are considered borderline. The standardised cell labelling, substrate preparation and adhesion protocol is summarised in Table 4.20.



**Table 4.20 Summary of the Standardised Cell Adhesion Assay.**

*Cell labelling procedure.*

1x10<sup>7</sup> cells incubated with 200kBq <sup>51</sup>Cr in 100µl for 1hr at 37°C

washed twice in 2ml assay medium (IMDM + 10% FCS)

10% aliquot retained for *total discard count*.

Resuspended in 2ml assay medium, cell count and viability checked by Neubauer chamber and trypan blue exclusion.

10% aliquot (200µl) is set aside (reserve)

*Substrate preparation.*

Stock solutions established: 0.1mg/ml collagens, 0.05mg/ml pFn and other glycoproteins / proteoglycans, 1% BSA.

100µl aliquots of stock incubated in each test well (24 well plate) for 1hr at room temperature.

Wells are washed with assay medium before use.

Each assay and control is carried out in triplicate wells.

*Adhesive phase.*

Aliquots of 1-2x10<sup>5</sup> cells in 400µl of assay medium are introduced to each test and control well.

Incubation for 2hrs at 37°C. Plates are wrapped in cellophane to prevent dehydration of the wells.

*Distractive phase.*

Supernatant is removed to an assay tube, and wells are washed twice with 200µl assay medium, with gentle shaking of the plate by hand each time. Addition and removal of media is carried out by 200µl pipette down the side of the well.



### *Results.*

Supernatant and washes (800µl) comprise the *non-adherent count*

Adherent cells are lysed by 15min with 500µl 0.1% Non-Idet in distilled water, and are then removed to a scintillation vial (*adherent count*)

The reserve cells are spun-out, and the supernatant (10% of the *total supernatant count*) and cells (10% of the *total cell count*) counted separately.

### *Analysis.*

Adherent and non-adherent samples for each well are counted sequentially by gamma counter.

Results are calculated as  $\text{adherent count} / \text{adherent} + \text{non-adherent count} (\%)$ , and the mean of triplicate wells is quoted.

Comparisons are made using paired or non-paired *t* test as appropriate.

**CHAPTER 5. APPLICATION OF THE  $^{51}\text{CHROMIUM}$  ADHESION ASSAY  
TO THE STUDY OF HUMAN HAEMATOPOIETIC CELL LINE  
ADHESION TO NON-CELLULAR SUBSTRATES.**

**5.1 Introduction.** **p 217**

**5.2 Study of the Adhesion of Human Haematopoietic Cell Lines to  
Extracellular Matrix Components.** **p 217**

**5.3 Effects of Activating Agents on KG1a adhesion to Extracellular Matrix  
Components.** **p 227**

**5.4 Detailed Studies of KG1a Binding to Fibronectin.** **p 232**

*.1 Background. p 232.*

*.2 A direct comparison of KG1a binding to plasma and tissue-derived  
fibronectin. p 234.*

*.3 Studies on the metabolic nature of KG1a adhesion to fibronectin. p 234.*

*.4 Blockade of KG1a-fibronectin adhesion by RGD-containing peptides. p 234.*

*.5 Blockade of KG1a-fibronectin adhesion by monoclonal antibodies. p 237.*

*.6 Blockade of KG1a-fibronectin adhesion by enzymatic treatment. p 241.*

**5.5 Discussion.** **p 243.**

### **5.1. Introduction.**

It was decided to study the adhesion of a representative group of human haematopoietic cell lines to various proteins and proteoglycans, stromal and endothelial cultures, in order to establish whether differences in lineage differentiation or CAM expression could be correlated with differences in functional adhesion. The cell lines KG1a, K562, HL60, CEM and NALM-6 were studied. The tissue culture of these cell lines in suspension is discussed in Section 2.2.2 and key characteristics summarised in Table 2.1. Lineage marker and cell adhesion molecule expression on these lines was characterised using single colour immunocytometry (Chapter 3.4), employing the same panel of mAbs used to characterise HPC in Chapter 3.2. It is acknowledged that some or all of these cell lines may express other CAMs, but this possibility could not be explored without the use of a very broad panel of anti-CAM mAbs, and such an approach was outwith the financial constraints of the study. Adhesion of these cell lines to a panel of extracellular matrix components (ECM) was studied using the  $^{51}\text{Cr}$  adhesion assay developed in Chapter 4 (Sections 5.2 and 5.3). Commercial thrombospondin was found to be prohibitively expensive and only KG1a adhesion to this substrate could be investigated. The nature of KG1a binding to fibronectin was examined in more depth in Section 5.4 using a combination of metabolic blockade, synthetic peptides, monoclonal antibodies, and enzymatic treatment. The composite results of these studies are discussed in Section 5.5.

### **5.2 Study of the Adhesion of Human Haematopoietic Cell Lines to Extracellular Matrix Components.**

The collagens, glycoproteins and proteoglycans studied were those identified by other groups as expressed within the bone marrow stroma [Bentley, 1982; Keating, Wright, Kinsell *et al.* 1984; Gordon, 1988a; Zuckerman, Prince & Gay, 1989; Clark, Gallagher & Dexter, 1992; Morris, Turnbull, Riley *et al.* 1991; Chichester, Fernandez & Minguell, 1993]. The study material was derived from commercial sources (Table 5.1), and therefore was not itself of human bone marrow stromal

**Table 5.1 Extracellular Matrix Components Studied.**

collagen type I (human placenta)
collagen type III (human placenta)
collagen type IV (human placenta)
fibronectin (human plasma)
fibronectin (human foreskin fibroblasts)
vitronectin (human plasma)
laminin (human placenta)
thrombospondin (human platelets)
heparan sulphate (bovine kidney)
chondroitin sulphate (bovine trachea)
hyaluronic acid (human umbilical cord)

---

origin. No source of hemonectin could be found [Campbell, Wicha & Long, 1987].

Stock solutions of collagens were made up at a concentration of 0.1mg/ml in 0.1% acetic acid in distilled water, and of fibronectin, vitronectin, thrombospondin, laminin and proteoglycans at a concentration of 0.05mg/ml in distilled water. 24 well tissue culture plates (surface area 2cm<sup>2</sup>) were layered with 100µl of test solution, and incubated at 37°C for 1hr. This gave a substrate concentration of 5µg / 50µl / cm<sup>2</sup> for collagens, and 2.5µg / 50µl / cm<sup>2</sup> for other proteins and proteoglycans. Triplicate wells were established for each test substrate, along with triplicate pFn and denatured dBSA wells as positive and negative controls respectively. The mean % adherence was derived and binding compared to negative control by two-tailed paired *t* test over a series of 4 replicate experiments. In view of the possibility that residual acetic acid in the collagen wells may alter the binding of the test cells through alteration in the pH of the assay medium, all wells were incubated with 400µl of assay medium for 5min prior to use, and this was removed prior to addition of the cells. Although increased acidity was frequently observed through change in the colour of this wash medium, no change was observed during

the test incubation itself. With the exception of this modification, no other change was made to the standardised protocol (Table 4.20).

Cells were  $^{51}\text{Cr}$  labelled using standard method, though the  $^{51}\text{Cr}$  uptake and labelling index did vary to some extent between cell lines (Table 5.2).

The results of this large series of experiments are summarised in Tables 5.3 - 5.7. KG1a appeared to bind to collagen type I, though this did not achieve statistical significance. KG1a bound significantly to fibronectin ( $p < 0.02$ ) and to thrombospondin ( $p < 0.001$ ) but not to vitronectin, laminin or the proteoglycans tested. K562 and HL-60 bound to fibronectin ( $p < 0.01$  and  $p < 0.02$  respectively) and appeared to bind to vitronectin and laminin though this was not statistically significant. These two lines did not adhere to the collagens or proteoglycans tested. NALM-6 bound strongly to fibronectin ( $p < 0.01$ ) and also bound to heparan sulphate ( $p < 0.05$ ), chondroitin sulphate ( $p < 0.01$ ) and to a lesser extent to hyaluronic acid (ns). Vitronectin and laminin binding were once again apparent but not statistically significant. CEM showed borderline binding to collagens type I (ns) and III (ns), and significant binding to fibronectin ( $p < 0.05$ ) and laminin ( $p < 0.01$ ). Adhesion to proteoglycans was also apparent, though the standard deviations were wide and the data not statistically significant. Some cell lines appear to demonstrate less binding to substrates than to dBSA *e.g.* KG1a to collagen IV and proteoglycans, K562, HL-60 and NALM-6 to collagens, although only in one case did this effect approach significance (Table 5.5). Background binding to dBSA did not differ significantly between cell lines (non-paired *t* test).

Clearly differences in functional binding of haematopoietic cell lines to ECM components do occur and some correlations with cell adhesion molecule expression are apparent (Figures 3.9 - 3.12 & Tables 5.3 - 5.7). For example, all five cell lines express VLA-4 (CD49d) and VLA-5 (CD49e) and all demonstrated significant binding to pFn. KG1a express CD36 and bind markedly to thrombospondin. Whether the lack of CD36 expression by the other cell lines correlated with absence of thrombospondin binding is unknown. However, important areas of discordance also occur. For example PECAM-1 (CD31) and CD45 expression by KG1a did not

**Table 5.2  $^{51}\text{Cr}$  Labelling of Haematopoietic Cell Lines.**

<i>cell line</i>	<i><math>^{51}\text{Cr}</math> uptake</i>	<i>labelling index</i>	<i>supernatant contamination (n)</i>
KG1a	$0.31 \pm 0.11$	$35 \pm 7$	$2.6 \pm 2.0\%$ (6)
K562	$0.48 \pm 0.07$	$44 \pm 13$	$6.0 \pm 5.6\%$ (4)
HL60	$0.56 \pm 0.17$	$62 \pm 7$	$1.2 \pm 0.5\%$ (4)
NALM-6	$0.31 \pm 0.16$	$16 \pm 3$	$10.1 \pm 6.6\%$ (4)
CEM	$0.28 \pm 0.18$	$13 \pm 7$	$7.4 \pm 8.3\%$ (4)

*Legend.* Results presented are the mean  $\pm$  standard deviation of 4 replicate experiments. Some cell lines did appear to label more easily than others. *e.g.* HL-60 has a high labelling index and low supernatant contamination compared with NALM-6.



**Table 5.3 Adhesion of KG1a to Extracellular Matrix Components.**

*Collagens.*

type I	25.8 ± 19.1% (ns)
type III	12.1 ± 7.6% (ns)
type IV	3.7 ± 1.9% (ns)

*Glycoproteins.*

plasma fibronectin.	28.2 ± 6.0% (p<0.02)
vitronectin.	11.7 ± 11.7% (ns)
thrombospondin	76.2 ± 12.8% (p<0.001)
laminin.	12.6 ± 11.7% (ns)

*Proteoglycans.*

heparan sulphate	4.4 ± 1.0% (ns)
chondroitin sulphate	5.2 ± 1.7% (ns)
hyaluronic acid	6.8 ± 1.3% (ns)

*Controls.*

dBSA.	9.6 ± 4.8%
-------	------------

*Legend.* Results are quoted as mean % adherence ± standard deviation of 4 replicate experiments. Substrates are compared with negative control (dBSA) by paired *t* test. Within each experiment, test substrates and controls were assayed in triplicate, and the mean % adherence used as the assay result. KG1a were found to bind significantly to plasma fibronectin and thrombospondin, and also bound to collagen type I (though non-significant due to a wide standard deviation).

**Table 5.4 Adhesion of K562 to Extracellular Matrix Components.**

*Collagens.*

type I	6.7 ± 1.4 (ns)
type III	7.6 ± 2.5 (ns)
type IV	5.0 ± 2.7 (ns)

*Glycoproteins.*

plasma fibronectin.	38.7 ± 4.1 (p<0.01)
vitronectin.	23.3 ± 3.1 (ns)
laminin.	38.1 ± 9.0 (ns)

*Proteoglycans.*

heparan sulphate	15.2 ± 15.0 (ns)
chondroitin sulphate	16.4 ± 17.1 (ns)
hyaluronic acid	12.3 ± 9.1 (ns)

*Controls.*

dBSA.	8.5 ± 3.0
-------	-----------

*Legend.* Results are quoted as mean % adherence ± standard deviation of 4 replicate experiments. Substrates are compared with negative control (dBSA) by paired *t* test. Within each experiment, test substrates and controls were assayed in triplicate, and the mean % adherence used as the assay result. K562 adhered significantly to plasma fibronectin only. Vitronectin and laminin adherence occurred but did not achieve statistical significance.

**Table 5.5 Adhesion of HL60 to Extracellular Matrix Components.**

*Collagens.*

type I	3.6 ± 3.3 (ns)
type III	5.4 ± 3.0 (ns)
type IV	2.9 ± 1.9 (p<0.05)

*Glycoproteins.*

plasma fibronectin.	30.7 ± 9.9 (p<0.02)
vitronectin.	41.8 ± 31.7 (ns)
laminin.	55.2 ± 20.3% (ns)

*Proteoglycans.*

heparan sulphate	8.4 ± 6.5 (ns)
chondroitin sulphate	7.6 ± 4.0 (ns)
hyaluronic acid	17.4 ± 9.8 (ns)

*Controls.*

dBSA.	12.1 ± 6.3
-------	------------

*Legend.* Results are quoted as mean % adherence ± standard deviation of 4 replicate experiments. Substrates are compared with negative control (dBSA) by paired *t* test. Within each experiment, test substrates and controls were assayed in triplicate, and the mean % adherence used as the assay result. HL-60 adhered significantly to fibronectin, and also to vitronectin and laminin - though this was not statistically significant due to the wide standard error of the results. Adhesion to collagen type IV was significantly less than that to dBSA.

**Table 5.6 Adhesion of NALM-6 to Extracellular Matrix Components.**

*Collagens.*

type I	10.0 ± 5.5% (ns)
type III	11.6 ± 6.7% (ns)
type IV	8.4 ± 5.3% (ns)

*Glycoproteins.*

plasma fibronectin.	82.1 ± 13.1% (p<0.01)
vitronectin.	37.0 ± 9.9 (ns)
laminin.	68.9 ± 20.7 (ns)

*Proteoglycans.*

heparan sulphate	41.6 ± 15.0% (p<0.05)
chondroitin sulphate	40.4 ± 7.9% (p<0.01)
hyaluronic acid	29.8 ± 11.6% (ns)

*Controls.*

dBSA.	14.6 ± 3.3
-------	------------

*Legend.* Results are quoted as mean % adherence ± standard deviation of 4 replicate experiments. Substrates are compared with negative control (dBSA) by paired *t* test. Within each experiment, test substrates and controls were assayed in triplicate, and the mean % adherence used as the assay result. NALM-6 adhered significantly to fibronectin, heparan sulphate and chondroitin sulphate, and also to vitronectin, laminin and hyaluronic acid.

**Table 5.7 Adhesion of CEM to Extracellular Matrix Components.**

*Collagens.*

type I	18.8 ± 5.1% (ns)
type III	21.9 ± 7.3% (ns)
type IV	12.0 ± 2.9% (ns)

*Glycoproteins.*

plasma fibronectin.	38.0 ± 13.3% (p<0.05)
vitronectin.	22.3 ± 2.8 (ns)
laminin.	72.4 ± 17.6% (p<0.005)

*Proteoglycans.*

heparan sulphate	29.8 ± 25.7% (ns)
chondroitin sulphate	30.7 ± 25.3% (ns)
hyaluronic acid	24.8 ± 19.1% (ns)

*Controls.*

dBSA.	9.6 ± 1.1
-------	-----------

*Legend.* Results are quoted as mean % adherence ± standard deviation of 4 replicate experiments. Substrates are compared with negative control (dBSA) by paired *t* test. Within each experiment, test substrates and controls were assayed in triplicate, and the mean % adherence used as the assay result. CEM adhered significantly to fibronectin and laminin and demonstrated borderline adhesion to collagens type I and III.

correlate with binding to heparan sulphate [Parish, Hogarth & McKenzie, 1988], whereas CD31<sup>+</sup> CD45<sup>+</sup> NALM-6 did adhere to this proteoglycan. Similarly, KG1a, HL60 and CEM express HCAM (CD44) but adhere neither to collagens nor to hyaluronic acid. K562 express VNR (CD51) though they do not appear to significantly bind to vitronectin. The functional activity of some of the other expressed CAMs could not be assessed because of the absence of the appropriate purified ligand *e.g.* ICAM-1 (CD54) (ligand LFA-1), PECAM-1 (CD31) (ligand PECAM-1), LFA-3 (CD58) (ligand LFA-2), LFA-1 (CD11a) (ligand ICAM-1) and VLA-4 (CD49d) (ligand VCAM-1).

The information required to correlate CAM expression and functional binding activity is incomplete. Part of the problem is that a complete characterisation of CAM expression by the cell lines studied has not been carried out and additional molecules may therefore be participating in cell adhesion. For example the CAM or CAMs which mediate CEM adhesion to laminin were not identified. In contrast, CAM expression is not sufficient in itself to convey adhesion to an appropriate ligand within the assay system. Some CAMs may be expressed in a non-activated form or may mediate adhesion to other, as yet unidentified, ligands. Alternatively, the purified ECM components used in this study may be comprised of the wrong isoforms or glycosylation pattern, or may have been presented in an unacceptable format. Finally some CAM-ligand binding is of low avidity and may be obscured by the relatively high shear stress imparted during the washing procedures (Chapter 4.4 & 4.5). Two approaches were taken to extend these observations. First, a phorbol ester and a calcium ionophore were used to attempt to "activate" cells in order to establish whether some CAMs were expressed in a non-activated state (Section 5.3). Second, a variety of approaches were used to attempt to blockade KG1a adhesion to pFn as a model system in which to establish whether a correlation between CAM expression and functional binding to the appropriate ligand was sufficient to conclude a causal link between the two (Section 5.4).



### 5.3 Effects of Activating Agents on KG1a Adhesion to Extracellular Matrix Components.

Integrin adhesiveness is known to be under tight regulatory control, with the ability to switch rapidly between multiple adhesive states in response to appropriate stimuli and intracellular signal transduction [Hogg, Bennett, Cabanas *et al.* 1992; Matsumoto & Hemler, 1993; Diamond & Springer, 1994]. For example, in T cells integrin activation occurs in response to cross-linking (ligation) of specific surface molecules (such as CD3 and CD7) or pharmacological agents such as the calcium ionophore A23187, and the phorbol ester - phorbol myristate acetate (PMA), through calcium mobilisation and protein kinase C activation respectively [Mobley, Ennis & Shimizu, 1994]. In these cells VLA-4 and VLA-5 mediated fibronectin adhesion is enhanced without alteration in intensity of cell surface expression [Ferreira, Valinsky, Sheridan *et al.* 1991; Uchiyama, Barut, Chauhan *et al.* 1992], and is therefore a result of conformational change in the molecule and / or clustering of integrins in the membrane. In HPC it is unknown whether CAMs are expressed in a non-activated or partially-activated form and if so what the stimuli and intracellular signalling mechanisms involved might be.

1µg/ml A23187 or 10ng/ml PMA in IMDM 10% FCS were added to KG1a following <sup>51</sup>Cr labelling, and incubated for 30min before aliquots were dispensed at the same concentration into the test wells. KG1a adherence to the panel of extracellular matrix components was examined (Table 5.8 & 5.9) and the results compared both to internal pFn and dBSA controls using a two-tailed paired *t* test, and to the studies on non-activated cells using a two-tailed non-paired *t* test. The results with A23187 were largely unremarkable, with no significant differences from baseline. The results with PMA were very interesting in that there was an apparent global increase in binding, with a non-significant increase in collagen binding, augmentation of fibronectin binding ( $p<0.001$ ), and significant increases in vitronectin ( $p<0.05$ ), laminin ( $p<0.01$ ), proteoglycans ( $p<0.05$ ) and dBSA binding ( $p<0.01$ ). Concomitant controls (not exposed to PMA) showed a small increase in fibronectin binding ( $p<0.05$ ) but no difference in dBSA binding comparative to the

**Table 5.8 Effects of A23187 on KG1a Adhesion to Extracellular Matrix Components.**

<i>ECM component</i>	<i>mean % adherence ± SD</i>	<i>significance cf: dBSA (paired t test)*</i>	<i>significance cf: non- activated cells (non-paired t test)<sup>s</sup></i>
<i>Collagens</i>			
Type I	3.4 ± 1.2%	ns (n=4)	ns (n=4)
Type III	4.4 ± 2.7%	ns (n=4)	ns (n=4)
TypeIV	1.9 ± 0.2%	p<0.05 (n=4)	ns (n=4)
<i>Glycoproteins</i>			
pFn	20.4 ± 14.0%	ns (n=4)	ns (n=4)
Vitronectin	9.1 ± 2.1%	(n=2)	ns (n=2)
Laminin	7.0 ± 3.6%	ns (n=4)	ns (n=4)

*Legend. next page.*

**Table 5.8 continued.**

<i>ECM component</i>	<i>mean % adherence ± SD</i>	<i>significance cf: dBSA (paired t test)*</i>	<i>significance cf: non- activated cells (non-paired t test)<sup>s</sup></i>
<i>Proteoglycans</i>			
Heparan sulphate	7.0 ± 5.0%	ns (n=4)	ns (n=4)
Chondroitin sulphate	8.8 ± 6.3%	ns (n=4)	ns (n=4)
Hyaluronic acid	8.4 ± 5.1%	ns (n=4)	ns (n=4)
dBSA	8.4 ± 7.0%	ns (n=4)	ns (n=4)
<i>Controls.</i>			
pFn	30.6 ± 11.4%	p<0.05 (n=4)	ns (n=4)
dBSA	9.1 ± 4.0%	ns (n=4)	

*Legend.* Results are reported as the mean ± standard deviation of 4 replicate experiments. Adhesion of activated KG1a to substrate is compared to that to dBSA by paired *t* test\*, and is compared to the adhesion of non-activated KG1a to dBSA by non-paired *t* test<sup>s</sup>. Activation of KG1a with calcium ionophore A23187 had no effect on adhesion to the panel of substrates.

**Table 5.9 Effects of 10ng/ml PMA on KG1a Adhesion to Extracellular Matrix Components.**

<i>ECM component</i>	<i>mean % adherence ± SD</i>	<i>significance cf: dBSA (paired t test)*</i>	<i>significance cf: non- activated cells (non-paired t test)<sup>s</sup></i>
<i>Collagens</i>			
Type I	34.6 ± 27.9%	ns (n=3)	ns (n=4)
Type III	34.1 ± 21.4%	ns (n=3)	ns (n=4)
Type IV	11.8 ± 8.1%	ns (n=3)	ns (n=4)
<i>Glycoproteins</i>			
pFn	83.4 ± 14.0%	p<0.05 (n=3)	p<0.001 (n=4)
Vitronectin	59.6 ± 25.0%	(n=2)	p<0.05 (n=3)
Laminin	67.8 ± 23.3%	ns (n=3)	p<0.01 (n=4)

*Legend. next page.*

**Table 5.9 continued.**

<i>ECM component</i>	<i>mean % adherence ± SD</i>	<i>significance cf: dBSA (paired t test)*</i>	<i>significance cf: non-activated cells (non-paired t test)<sup>s</sup></i>
<i>Proteoglycans</i>			
Heparan sulphate	55.8 ± 28.2%	ns (n=3)	p<0.05 (n=3)
Chondroitin sulphate	56.9 ± 23.0%	ns (n=3)	p<0.05 (n=3)
Hyaluronic acid	57.9 ± 22.7%	ns (n=3)	p<0.05 (n=3)
dBSA	60.4 ± 26.4%	ns (n=3)	p<0.01 (n=4)
<i>Controls.</i>			
pFn	44.2 ± 9.9%	p<0.05 (n=3)	p<0.05 (n=4)
dBSA	7.9 ± 2.8%	ns (n=3)	

*Legend.* Results are reported as the mean ± standard deviation of 4 replicate experiments. Adhesion of activated KG1a to substrate is compared to that to dBSA by paired *t* test\*, and is compared to the adhesion of non-activated KG1a to dBSA by non-paired *t* test<sup>s</sup>. A global increase in KG1a binding to substrates and dBSA control occurs following activation with PMA.

baseline investigation implying that overall technique in terms of washing stringency and dBSA batch were similar.

This series of experiments and those preceding, generated two vexed questions: First, is PMA having multiple effects on integrin and non-integrin CAMs, or is there a negative global regulator of adhesion (such as ionic charge) which is reduced by exposure to PMA? Second, is the assumption that dBSA comprises a "neutral" background valid? How should the observations that binding to proteoglycans and collagens (Tables 5.3 -5.7) is apparently less than that to dBSA, and the marked increase in dBSA binding with PMA stimulation (Figure 5.9), be interpreted?

## **5.4 Detailed Studies of KG1a Adhesion to Fibronectin.**

### *5.4.1 Background.*

Fibronectin fulfils a major role in maintaining the stability of the ECM in the haematopoietic stroma, and long-term marrow cultures (*vide infra*) [Singer, Keating & Wight, 1985]. It is a dimeric molecule, containing domains for binding of other ECM proteins such as fibrin, collagens, and proteoglycans such as heparin and heparan sulphate [Hynes, 1990; Schwarzbauer, 1991]. It contains at least two discrete cell binding domains: an arginine-glycine-aspartic acid (RGD) containing cell binding domain in the central part of the molecule - a ligand for VLA-5 (CD49e/CD29) [Ruoslahti & Pierschbacher, 1987], and a proline-gluamic acid-isoleucine- leucine-aspartic acid-valine (PEILDV) containing domain at the carboxy-terminal adjacent to the high-affinity heparin binding domain (the CS1 alternatively spliced domain) - which is a ligand for VLA-4 (CD49d/CD29). The vitronectin receptor (CD51/CD61) and platelet glycoprotein IIbIIIa (CD41a/CD61) are also known to be fibronectin receptors [Uchiyama, Barut, Chauhan *et al.* 1992], as are other integrin vitronectin receptors such as  $\alpha_v \beta_1$  and  $\alpha_v \beta_3$  (Table 1.9). In addition, the integral membrane proteoglycan syndecan, binds to the heparin-binding domain of fibronectin via its heparan sulphate side chains [Saunders & Bernfield, 1988]. Fibronectin may play a pivotal role in the co-localisation of proteoglycans, cytokines and haematopoietic cells within the stroma. At least 20



variants have been described, which arise through alternative mRNA splicing involving extra-domains a and b (EDa and EDb) and / or the carboxy-terminal variable region. To some extent, fibronectin isoforms are cell type specific, and may have different functions [Schwarzbauer, 1991]. Lerat, Lissitzky, Singer *et al.* [1993] have shown that vascular smooth muscle-like stromal cells in human bone marrow stromal cultures synthesise and secrete an EDa<sup>+</sup> EDb<sup>-</sup> fibronectin variant, which is incorporated into the ECM. Granulo-monocytic cells secreted an EDa<sup>-</sup> EDb<sup>-</sup> variant, similar to that found in normal human and horse plasma [Tressel, McCarthy, Calaycay *et al.* 1991], which shows poor ECM incorporation.

Both normal HPC, and the haematopoietic cell lines studied, express VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29), though not the vitronectin receptor (CD51/CD61) nor glycoprotein IIbIIIa (CD41/CD61) (Figures 3.2 & 3.3, 3.5 - 3.12). Negligible binding to vitronectin makes a contribution from the alternative receptors  $\alpha_v\beta_1$  and  $\alpha_v\beta_5$  unlikely. Tavassoli *et al.* have demonstrated that membrane-bound chondroitin sulphate can contribute to HPC-fibronectin binding [Minguell, Hardy & Tavassoli, 1992].

CAM blockade can be effected by non-specific mechanisms such as divalent cation chelation (which abrogates integrin and selectin binding), by semi-specific mechanisms such as synthetic RGD-containing peptides or enzymatic treatment, or by highly-specific mechanisms such as mAbs which address the binding domain of the CAM or alter the conformational structure of the molecule.

It was demonstrated in Chapter 4.3.3 that use of an assay medium free of divalent cations (HBSS + 10mM EDTA) reduces KG1a binding to pFn (Table 4.8), suggesting that this process is predominantly integrin dependent. The effects of divalent cations may be differential, studies on LFA-1 have suggested that leucocyte integrin activity is augmented by  $Mn^{2+}$  and  $Mg^{2+}$  but inhibited by  $Ca^{2+}$  [Dransfield, Cabanas, Craig *et al.* 1992]. A series of studies was carried out to analyse the nature of KG1a adhesion to Fn in more depth.

#### *5.4.2 A direct comparison of KG1a binding to plasma and tissue-derived fibronectin.*

KG1a adhesion to both plasma-derived (pFn) and tissue-derived fibronectin (tFn) was compared in a discrete series of experiments on the grounds that potential differences in the balance of Fn isoforms may contribute to functional differences in fibronectin derived from different sources [Lerat, Lissitzky, Singer *et al.* 1993]. The results are presented in Table 5.10. tFn displayed higher binding of KG1a than pFn ( $p < 0.05$ ), and both displayed significant binding compared to dBSA background ( $p < 0.001$  both). This suggested that tFn displayed more ligands or different kinds of ligands compared to pFn.

#### *5.4.3 Studies on the metabolic nature of KG1a adhesion to plasma fibronectin.*

To assess the metabolic requirements of adhesion to fibronectin, KG1a were incubated with  $^{51}\text{Cr}$  in the usual manner, washed, distributed into appropriate aliquots, and incubated for 1 hr at ambient temperature with 30 mmol/l of sodium fluoride, 10  $\mu\text{g/ml}$  of cytochalasin B, or 1  $\mu\text{g/ml}$  of cycloheximide, in RPMI 10% FCS. Cells were re-distributed to individual triplicate wells, and incubated with pFn in medium containing the same concentration of the appropriate compound. Sodium fluoride depletes cellular adenosine triphosphate [Narayanan, Su & Bedard, 1991], cytochalasin B paralyses the cytoskeleton by blocking the assembly of actin filaments, and cycloheximide inhibits protein synthesis [Majdic, Stockl, Pickl *et al.*, 1994]. The results of this study are summarised in Table 5.11. None of these manipulations had any significant effect on KG1a-pFn adhesion, suggesting that the latter is a feature of constitutively expressed and activated cell adhesion molecules.

#### *5.4.4 Blockade of KG1a-fibronectin adhesion by RGD-containing peptides.*

The recognition of a number of ECM proteins by integrins is mediated through a common arginine-glycine-aspartic acid (RGD) containing domain [Ruoslahti & Pierschbacher, 1986 & 1987]. VLA-4 (CD49d/CD29) recognition of fibronectin is mediated through a separate domain, and is not RGD-blocked (*vide supra*). The

**Table 5.10 Comparative Adhesion of KG1a to Plasma and Tissue Fibronectin.**

<i>substrate</i>	<i>mean % adherence</i>	<i>significance (n)</i>
pFn	68.1 ± 9.2%	p<0.001 (5)
tFn	74.0 ± 7.7%	p<0.001 (5)
dBSA	28.8 ± 11.4%	(5)

*Legend.* Data is presented as the mean ± standard deviation of 5 replicate experiments. Statistical significance is calculated comparative to dBSA (paired *t* test). Adhesion to tFn proved greater than that to pFn (p<0.05) (paired *t* test).

**Table 5.11 Effects of Metabolic Inhibitors on KG1a Adhesion to Plasma Fibronectin.**

<i>Medium (substrate)</i>	<i>mean % adherence</i>	<i>cf. pFn*</i>	<i>cf. dBSA<sup>s</sup></i>
sodium fluoride (pFn)	44.4 ± 14.2%	ns	p<0.01
cytochalasin B (pFn)	46.6 ± 24.0%	ns	p<0.05
cycloheximide (pFn)	63.7 ± 11.0%	ns	p<0.001
RPMI 10% FCS (pFn)	48.2 ± 6.0%		p<0.01
RPMI 10% FCS (dBSA)	15.2 ± 10.5%		

*Legend.* Data is presented as the mean ± standard deviation of 4 replicate experiments. Adhesion of KG1a to pFn in the presence of metabolic inhibitors is compared to that in normal medium\* and to background<sup>s</sup> by two-tailed paired *t* test. No significant effect of the metabolic inhibitors studied could be demonstrated.

importance of VLA-5 in fibronectin adhesion can therefore be explored using synthetic peptides.

KG1a were  $^{51}\text{Cr}$  labelled using standard technique and washed twice in assay medium. Aliquots containing  $6 \times 10^5$  cells were re-suspended in IMDM containing 1-10mM arginine-glycine-aspartic acid-serine (RGDS) peptide or arginine-glycine-glutamic acid-serine (RGES) peptide as control. In two separate series of experiments, adhesion to pFn and to tFn was studied in this way. The results are summarised in Table 5.12. Inhibition of binding by RGES appeared to be independent of peptide concentration. Inhibition by RGDS was highly concentration dependent, and reached a plateau at 5mM. Significant differences between RGDS and RGES inhibition were present at 2.5mM ( $p < 0.05$ ) and 5mM ( $p < 0.01$ ) only (paired  $t$  test). Study of KG1a binding to tFn was carried out in a separate series of experiments, and a very similar pattern of inhibition was demonstrated. A two-tailed non-paired  $t$  test showed no demonstrable difference between inhibition of adhesion to pFn and tFn. A heptapeptide containing additional residues immediately adjacent to the RGD sequence in fibronectin (glycine-arginine-glycine-aspartic acid-serine-proline-cysteine) has been shown to be better at inhibiting attachment of cells to fibronectin and vitronectin [Gartner & Bennett, 1985; Pierschbacher & Ruoslahti, 1987; Hautanen, Gailit, Mann *et al.* 1989].

#### *5.4.5 Blockade of KG1a-fibronectin adhesion by monoclonal antibodies.*

A further analysis of KG1a adhesion to pFn was undertaken using mAbs of known blockading ability. The anti- $\alpha 4$  mAb HP2/1 abrogates binding of human myeloma cell lines and T-lymphocytes to the CS1 domain of fibronectin [Uchiyama, Barut, Chauhan *et al.* 1992; Nojima, Humphries, Mould *et al.* 1990]. The anti- $\alpha 4$  mAb L25.3 and has been shown to inhibit T lymphocyte interaction with human endothelial cells, VCAM-1 transfected L cells, and Fn [Clayberger, Krensky, McIntyre *et al.* 1987]. mAb 16 has anti- $\alpha 5$  chain specificity, and has been shown to inhibit fibroblast attachment to Fn, but not to vitronectin or laminin [Akiyama, Yamada, Chen *et al.* 1989].

**Table 5.12 Blockade of KG1a Adhesion to Fibronectin by RGD-Containing Peptides.**

<i>Peptide</i>	<i>RGDS</i>	<i>cf: pFn</i>	<i>RGES</i>	<i>cf: pFn</i>
nil	31.9 ± 6.5%			
5mM	8.1 ± 1.5%	p<0.01	27.1 ± 6.8%	p<0.05
2.5mM	12.5 ± 1.2%	p<0.02	24.7 ± 6.0%	p<0.02
1mM	21.8 ± 6.7%	p<0.01	22.5 ± 3.4%	p<0.05
0.5mM	21.7 ± 6.4%	p<0.05	23.9 ± 7.2%	ns
dBSA	19.0 ± 11.3%			

*Legend.* Results are presented as mean % adherence ± standard deviation. A paired *t* test was used to compare % adhesion in the presence of synthetic peptides with adhesion to the fibronectin in the media alone (IMDM 10% FCS). Both peptides inhibited KG1a adhesion to pFn, but RGDS blockade increased with concentration and was more marked above 2.5mM.



In order to determine the appropriate quantity of mAb to be used in blockading experiments,  $5 \times 10^5$  KG1a were incubated with serial titrations of each mAb adjusted such that 10 $\mu$ g, 5 $\mu$ g, 1 $\mu$ g, 0.5 $\mu$ g, 0.1 $\mu$ g and 0  $\mu$ g was present in 10 $\mu$ l during the incubation step. These were stained with SAM-FITC second step reagent using standard immunocytometry protocol, and analysed by flow cytometry. The rationale of this approach was to achieve saturation of appropriate CAM epitopes, without a large excess of mAb which could cross-link and activate the study population. These titration experiments showed that 10 $\mu$ l containing 1 $\mu$ g of mAb (*i.e.* a concentration of 100 $\mu$ g/ml) was sufficient to achieve saturation binding, and is consistent with accepted practice [Coligan, Kruisbeek, Margulies *et al.* 1994]. Some authors have commented that sodium azide used as a preservative in mAb preparations is a metabolic inhibitor and membrane immobiliser and may interfere with cell adhesion. It proved unfeasible to obtain mAbs without sodium azide preservative, so a control was run with each series of experiments which included an incubation step with 10 $\mu$ l of PBS, 0.01% sodium azide.

KG1a were  $^{51}\text{Cr}$  labelled and washed using standard technique, and aliquots of  $5 \times 10^5$  cells were pelleted and incubated with 1 $\mu$ g of one of the three mAbs in 10 $\mu$ l PBS for 30min at +4°C. Controls were established with the same concentration of an IgG2a mAb of irrelevant specificity, with PBS 0.1% sodium azide, and without an incubation phase. The cells were resuspended in 1.2ml of culture medium, and triple aliquots of 400 $\mu$ l incubated with pFn for 2hrs in the standard manner. An adherent percentage was calculated and compared to controls by two-tailed paired *t* test.

The results of this series of experiments is presented in Table 5.13. Adhesion of KG1a was uniformly greater in those cell samples which had been incubated with mAb, including those in which an IgG2a of irrelevant specificity had been used, compared to those samples incubated without mAb (medium alone and with sodium azide). There was no clear difference between mAbs, with none showing significant reduction in adhesion compared to the IgG2a control. Adhesion in the presence of the sodium azide containing medium was similar to that in the positive control. All

**Table 5.13 KG1a Adhesion to Fibronectin in the Presence of Monoclonal Antibodies.**

<i>substrate</i>	<i>mAb</i>	<i>mean % adhesion</i>	<i>cf:dBSA*</i>	<i>cf:pFn<sup>s</sup></i>	<i>cf:IgG2a<sup>#</sup> (n)</i>
pFn	HP2/1	53.1 ± 23.6%	p<0.02	p<0.05	ns(n=3)
pFn	L25.3	45.0 ± 20.4%	p<0.05	ns	ns (n=4)
pFn	mAb16	51.9 ± 22.4%	p<0.01	p<0.05	ns (n=4)
pFn	IgG2a	55.8 ± 18.5%	p<0.001	p<0.01	(n=4)
pFn	sodium azide	35.6 ± 19.0%	p<0.05	ns	(n=4)
pFn	nil	35.7 ± 16.7%	p<0.01		(n=4)
dBSA	nil	21.1 ± 15.9%			(n=4)

*Legend.* Results are reported as mean ± standard deviation of 4 replicate experiments. Statistical significance of observed values were compared to dBSA\*, pFn<sup>s</sup> and pFn following incubation with IgG2a<sup>#</sup> using paired *t* test. The mAbs studied did not effect blockade of KG1a-pFn adhesion, indeed a paradoxical increase in adhesion was demonstrated, which was dependent on the presence but not the specificity of a mAb, and could not be ascribed to sodium azide.

samples were superior to the negative (dBSA) control. The results of this series of experiments were unexpected, and not easy to interpret. It seemed clear that the effect of the mAbs on KG1a-pFn adhesion was not a feature of antibody specificity, but nevertheless required the presence of the mAb since the presence of handling medium containing sodium azide alone did not effect KG1a-pFn adhesion. The cause of this effect is unclear. Cell aggregation did not occur, but the presence of the immunoglobulin could have caused CAM activation via Fc receptor occupancy or cross-linking, or may have acted as a molecular bridge between pFn and the cell. The absence of an expected blockading effect in the presence of relevant mAbs is difficult to interpret in view of this artefact. A repeat series of experiments following Fc blockade with Fab' or F(ab')<sub>2</sub> fragments may help to resolve the problem.

#### *5.4.6 Blockade of KG1a-fibronectin adhesion by enzymatic treatment.*

The groups based in Minneapolis [Verfaillie, Benis, Iida *et al.* 1994] and Jackson [Minguell, Hardy & Tavassoli, 1992; Conget & Minguell, 1994] have demonstrated cooperation between binding of a cell surface chondroitin sulphate (the core protein of which is related to CD44), and VLA-4 (CD49d/CD29) in binding to the heparin binding domain of Fn. To test the hypothesis that cell surface proteoglycans may be involved in HPC-Fn adhesion, KG1a were <sup>51</sup>Cr labelled in the usual manner and aliquots of 5x10<sup>5</sup> cells were incubated for 1hr at 37°C in 100µl of IMDM containing 0.1 units of chondroitinase ABC [Verfaillie, Benis, Iida *et al.* 1994], 0.5 units of heparinase [Coombe, Watt & Parish, 1994], 100 units hyaluronidase [Koshiishi, Shizari & Underhill, 1994] or with no added enzyme. A titration was not carried out, but the enzymes were used at concentrations which effectively blocked cell adhesion in the studies cited. The labelled cells were resuspended in the appropriate volume of medium and plated into triplicate wells with pFn (Table 5.14). KG1a adhesion to pFn was partially blocked by incubation with chondroitinase ABC, but not by heparinase or hyaluronidase.

**Table 5.14 Comparative Effects of Enzymatic Digestion of Proteoglycans on KG1a Adhesion to Fibronectin.**

<i>enzyme</i>	<i>mean % adhesion</i>	<i>cf: dBSA</i>	<i>cf: pFn</i>
Chondroitinase ABC	32 ± 5%	p<0.01	p<0.001
Heparinase	61 ± 2	p<0.01	ns
Hyaluronidase	61 ± 2	p<0.01	ns
pFn	59 ± 4	p<0.01	
dBSA	17 ± 6		

*Legend.* Data presented as the mean ± standard deviation of 4 replicate experiments. KG1a adhesion to pFn following enzyme incubation compared to that without and to dBSA by paired *t* test. Only Chondroitinase ABC demonstrated evidence of adhesion blockade.

## 5.5 Discussion.

The objective of the studies reported in this Chapter was to establish the extent to which phenotypic CAM expression could be correlated with functional cell adhesion to purified putative ligands. In addition, the utility of activating and blockading agents in modifying CAM function and cellular adhesion behaviour was explored in order to establish whether a positive correlation between the two was sufficient to infer a direct causal relationship.

It is clear that some correlations between CAM expression and ligand binding do exist. For example, all 5 cell lines express VLA-4 (CD49d) and VLA-5 (CD49e) and all demonstrated significant adhesion to pFn. However, there were also found to be areas of discordance, for example PECAM-1 (CD31) and CD45 expression by KG1a did not correlate with adhesion to heparan sulphate. On the grounds that some CAMs may therefore be expressed in a non- or partially-activated state, 2 reagents, the calcium ionophore A23187 and phorbol myristate acetate, were used as activating agents. Although A23187 had no effect, PMA caused an increase in adhesion to virtually all the substrates studied, raising the possibilities of a generalised activation of identified CAMs together with the presence of further, as yet unidentified, CAMs, or the downregulation of a global negative regulator of adhesion.

KG1a adhesion to pFn was examined in more depth to establish whether a positive correlation between CAM expression and adhesion behaviour could be taken to imply causality. Both divalent cation chelation and RGDS-containing peptides had a similar partial blockading effect on KG1a adhesion to pFn, confirming the involvement of VLA-5 in this interaction. Neither mAbs to VLA-4 nor to VLA-5 blocked KG1a-pFn adhesion suggesting either that there was an un-addressed methodological problem with this series of experiments, or that the antibodies used did not appropriately block this adhesion despite blockading VLA-4 and VLA-5 function in some other experimental systems. The involvement of VLA-4 in KG1a-pFn adhesion therefore remains moot. Moreover, the data from Section 5.4.6 clearly supports a corollary role for chondroitin sulphate, which would not

have been predicted from the immunofluorescence studies.

It is clear from these experiments, therefore, that CAM expression is an insufficient basis in itself to conclude involvement in cellular adhesion to an appropriate ligand, and moreover that the phenotypic characterisation of cellular CAM expression is incomplete, with the involvement of other classes of molecules such as proteoglycans. A variety of phenotypic and functional experiments are necessary to build up a complete picture of a particular cell-ligand interaction.

Two further limitations are inherent in this reductionist approach of studying haematopoietic cell line adhesion to purified ECM components: first that HPC interaction with the bone marrow microenvironment *in vivo* is likely to involve multiple cell-cell and cell-substrate adhesive pathways, only some of which can be replicated using purified components. Second, that the study population differs from normal human HPC. It was therefore felt to be important to investigate whether the adhesion assay could be extended to study cell adhesion to bone marrow stromal and endothelial tissue cultures *in vitro*, and whether HPC from human sources could be purified and  $^{51}\text{Cr}$  labelled in a satisfactory manner. These issues are addressed in Chapter 6.



## **CHAPTER 6. APPLICATION OF <sup>51</sup>CHROMIUM ADHESION ASSAY TO THE STUDY OF CELLULAR SUBSTRATES AND TO ENRICHED POPULATIONS OF HUMAN HAEMATOPOIETIC PROGENITORS.**

<b>6.1 Introduction.</b>	<b>p 246</b>
<b>6.2 Preparation of <i>in vitro</i> Tissue Cultures.</b>	<b>p 247</b>
.1 <i>Background.</i>	p 247.
.2 <i>Preparation of adherent cell lines.</i>	p 249.
.3 <i>Preparation of human fibroblast and stromal cultures.</i>	p 253.
<b>6.3 Preparatory Experiments to Study the Adhesion of KG1a to <i>in vitro</i> Tissue Cultures.</b>	<b>p 254</b>
<b>6.4 Comparative Adhesion of Haematopoietic Cell Lines to M210-B4.</b>	<b>p 260</b>
<b>6.5 Blockade of KG1a Adhesion to M210-B4.</b>	<b>p 262</b>
.1 <i>Blockade of KG1a adhesion by divalent cation chelation and synthetic peptides.</i>	p 262.
.2 <i>Blockade of KG1a adhesion by monoclonal antibodies.</i>	p 262.
.3 <i>Blockade of KG1a adhesion by enzymatic treatment.</i>	p 264.
.4 <i>Discussion.</i>	p 264.
<b>6.6 Development of a Haematopoietic Progenitor Enrichment Protocol.</b>	<b>p 267</b>
.1 <i>Background and definitions.</i>	p 267.
.2 <i>Preliminary experimental studies.</i>	p 271.
.3 <i>Effects of Ficoll-Hypaque gradient on CD34<sup>+</sup> cells.</i>	p 271.
.4 <i>The use of Dynal anti-CD34 beads and bead detachment systems.</i>	p 273.
.5 <i>The use of Miltenyi MACS system.</i>	p 277.
.6 <i>Sequential MACS and FACSorting enrichment protocol.</i>	p 281.
<b>6.7 Application of the <sup>51</sup>Cr Adhesion Assay to Highly Enriched HPC.</b>	<b>p 284</b>
.1 <i>Pilot studies of <sup>51</sup>Cr labelling of peripheral blood lymphocytes.</i>	p 284.
.2 <i><sup>51</sup>Cr labelling of highly enriched haematopoietic progenitors.</i>	p 286.
<b>6.8 Discussion.</b>	<b>p 287</b>
.1 <i>Long-term bone marrow culture as a model of <i>in vivo</i> events.</i>	p 287.
.2 <i>Is the <sup>51</sup>Cr adhesion assay applicable to haematopoietic progenitors?</i>	p 288.

## 6.1 Introduction.

To evaluate the relevance of the observations made using haematopoietic cell lines and highly purified extracellular matrix components to the interaction of HPC with the complex bone marrow endothelial and stromal environment *in vivo*, it was decided to extend the  $^{51}\text{Cr}$  adhesion assay in two ways: first to permit analysis of cell adhesion to bone marrow stromal culture layers *in vitro*, second to allow the study of HPC themselves.

Long-term tissue cultures allow the study of cell-cell interactions *in vitro* in a system that incorporates an order of complexity close to that exhibited by haematopoiesis *in vivo*. The preparation of adherent cell lines and bone marrow stromal cultures is discussed in Section 6.2. Haematopoietic cell lines were used to establish the feasibility of applying the  $^{51}\text{Cr}$  adhesion assay to stromal cultures for the same reasons of availability, consistency and characterisation outlined in Chapter 4.1 (Section 6.3). Comparative adhesion of haematopoietic cell lines to the murine stromal cell line M210-B4 (Section 6.4) were carried out, and the feasibility of carrying out blockading experiments within the assay system was explored (Section 6.5). In collaboration with Dr John Sweetenham and Mrs Lisa Masek of the CRC Medical Oncology Unit Southampton General Hospital, studies have been carried out to examine the adhesion of haematopoietic cell lines to human bone marrow endothelial cultures *in vitro*. The methodology and preliminary results of this work are discussed in Appendix 4.

It was clear that the application of the  $^{51}\text{Cr}$  adhesion assay to HPC required the use of highly enriched populations and a three stage enrichment protocol was developed with this in mind (Section 6.6). There are both advantages and disadvantages to the use of highly purified HPC in adhesion assays. To advantage, potential interference or alteration of HPC adhesion by accessory cells (for example: by competition for available binding sites, steric hindrance, or cytokine secretion), is avoided. To detriment, cell loss and functional alteration occurring during the enrichment process may be profound, and difficult to assess. Limitations in the number of purified HPC available for adhesion experiments made it desirable to

explore the possibility of increasing the labelling intensity of the target population (Section 6.7). The general applicability of the assay approach to HPC is constrained by the large starting population required, the extended nature of the enrichment protocol, and the intensity of the  $^{51}\text{Cr}$ -labelling procedure.

## **6.2 Preparation of *in vitro* Tissue Cultures.**

### **6.2.1 Background.**

Long term culture of bone marrow stromal layers (LTBMC) was first developed from mice by Dexter, Allen & Lajtha [1977], and later adapted to human marrow by Gartner and Kaplan [1980]. In this system, cells aspirated from bone marrow were maintained in an osmotically balanced culture medium and serum, and incubated at 33°C, 5% CO<sub>2</sub> and 100% humidity. An adherent layer formed over three to four weeks, which supported haematopoiesis in the absence of exogenous growth factors. Greenberger [1978] demonstrated that supplementation of the culture medium with corticosteroids stimulated lipid accumulation in stromal cells, and long-term proliferation of haematopoietic cells.

Stromal LTBMC preferentially support granulopoiesis, but it is clear that manipulation of the culture environment can lead to substantial alteration in the behaviour of the culture. For example, Whitlock and Witte [1982] have shown that B lymphopoiesis can be supported in murine LTBMC by alteration in the culture conditions. It is clear, therefore, that pluripotential haematopoietic stem and early progenitor cells adhere to LTBMC, and that their proliferation and differentiation is dependent on the culture environment.

In man it has been found that culturing bone marrow in a standard medium supplemented with FCS, leads to outgrowth of marrow fibroblasts which preferentially support lymphopoiesis, whilst supplementing the standard medium with horse serum and hydrocortisone, leads to formation of a morphologically more heterogeneous stromal population with adipocyte formation, which supports myelopoiesis (Dexter-type long term bone marrow stromal cultures). Unfortunately only 20-40% of horse sera will support adipocyte formation and these have to be

checked on a batch-batch basis. The confluent adherent layer which forms in LTBMCM after 3-4 weeks is a complex cellular and extracellular environment, which has only been partially characterised. Five stromal cell populations are identifiable: monocyte-macrophages, fibroblasts / reticular cells, "blanket cells", adipocytes and endothelial cells [Allen & Dexter, 1984; Riley & Gordon, 1987; Allen, Dexter & Simmons, 1990; Quesenberry, Temeles, McGrath *et al.* 1991]. These form a three dimensional structure with the endothelial cells forming an incomplete layer at the stromal / fluid interface [Allen & Dexter, 1982]. The relationship between reticular cells and fibroblasts is unclear. Fibroblasts in LTBMCM may be derived from adventitial reticular cells, from fibroblasts associated with vascular and nerve structures, or free lying fibroblasts within the marrow cavity. Charbord *et al.* have pointed out the phenotypic similarities between reticular cells and vascular smooth muscle cells based on data on studies of cytoskeletal proteins and on secretion of fibronectin isoforms [Charbord, Lerat, Newton *et al.* 1990; Galmiche, Koteliensky, Briere *et al.* 1993; Lerat, Lissitzky, Singer *et al.* 1993]. The origin of cells accumulating lipid in LTBMCM systems is equally uncertain. Tavassoli [1989] has pointed out that lipid accumulation *per se* does not warrant the classification of a cell as an adipocyte, since many cell types, including fibroblasts, adventitial reticular cells and macrophages, may accumulate lipid both *in vivo* and *in vitro*. In the absence of objective criteria with which to identify pre-adipocytes, it is unclear whether lipid accumulation *in vitro* reflects a culture phenomenon, or development of true marrow adipocytes. Although Greenberger demonstrated that addition of hydrocortisone to LTBMCM potentiated both lipogenesis and granulopoiesis [Greenberger, 1978], the interdependence of the two is debated [Allen & Dexter, 1982; Touw & Lowenberg, 1983]. Recent evidence suggests that the addition of steroids to LTBMCM leads to a decrease in stromal hyaluronic acid and a higher incorporation of sulphated glycosaminoglycans such as heparan sulphate. The extent to which LTBMCM can be said to be representative of bone marrow stromal structure *in vivo* remains uncertain.

From a functional point of view, long-term granulopoiesis occurs in LTBMCM in

the absence of exogenous growth factors, and is dependent on the presence of the confluent stromal layer [Allen & Dexter, 1982; Dexter, Coutinho, Spooncer *et al.* 1990; Verfaillie, Blakolmer & McGlave, 1990]. It appears that primitive HPC are preferentially integrated in the adherent layer, whereas with increasing maturity cells migrate to the surface and are shed into the growth medium [Mauch, Greenberger, Botnick *et al.* 1980; Toksoz, Dexter, Lord *et al.* 1980; Winton & Colenda, 1987]. In murine cultures, the addition of erythropoietin with agitation of the culture, leads to decreased lipid storage, regression of endothelial cells, and augmentation of erythropoiesis [Eliason, Testa & Dexter, 1979]. Similarly, modification of murine LTBMCM by use of low concentrations of FCS, addition of mercaptoethanol, and incubation at 37°C, promotes lymphopoiesis [Kincade, 1987]. In summary, these culture systems are probably the least artefactual *in vitro* model available for examining HPC-stromal adhesion mechanisms, but remain highly dependent on culture conditions and are therefore difficult to standardise.

The murine stromal cell line M210-B4 [Sutherland, Eaves, Lansdorp *et al.* 1991] was used as a convenient substrate for initial studies (Figure 6.1). No human stromal cell line could be found for study, though some have recently been described by Aizawa [Aizawa, Yaguchi, Nakano *et al.* 1991]. The validity of using a murine stromal cell line as representative of human bone marrow stroma is supported by experiments in which early human HPC are maintained for several weeks following infusion into immunodeficient mice [Zanjani, Pallavicini, Ascensao *et al.* 1992; Lapidot, Pflumio, Doedens *et al.* 1992] or when cocultured *in vitro* with murine stromal cells in the absence of added human growth factors [Sutherland, Eaves, Lansdorp *et al.* 1991; Issaad, Croisille, Katz *et al.* 1993; Burroughs, Gupta, Blazar *et al.* 1994].

#### 6.2.2. Preparation of adherent cell lines.

Adherent cell lines were cultured and passaged in 25cm<sup>3</sup> or 75cm<sup>3</sup> tissue culture flasks as previously described (Chapter 2.2.2). To prepare a 24 well plate for an adhesion assay, the adherent cells were removed from the primary culture flask and



**Figure 6.1 M210-B4 Murine Stromal Cell Line.**



*Legend.* M210-B4 murine bone marrow fibroblast cell line at confluence.  
(magnification x4 blue filter).



disaggregated by removing the culture medium, washing twice with 10ml of anticoagulant medium, and incubating with 5ml of trypsin / EDTA at 37°C. The cells were resuspended in culture medium at a concentration of  $2 \times 10^5$  cells / ml, and 0.5ml aliquots transferred to each well of the test plate. M210-B4 were found to grow to confluence in 2cm<sup>2</sup> wells within 48-72hrs, and thereafter were used as soon as possible. Senescent cultures (> 7 days) were found to deteriorate rapidly with fragmentation of the adherent layer. The morphology of a confluent adherent cell line at 3 days is illustrated in Figure 6.1. The use of smaller volumes of culture medium (especially less than 200µl) was found to lead to preferential growth of cells around the perimeter of the wells, and central subconfluence. This was thought to be a reflection of an unequal depth of culture medium in the well either due to a central convexity of the well base or (more likely) the effects of surface tension acting at the periphery (Chapter 4.4.3). A further technical problem was found to be the stability of the adherent layer in the face of recurrent addition and removal of media during the adhesion assay. M210-B4 were vulnerable to traumatic disruption, leading to loss of attachment at the periphery of the well (rolling) (Figure 6.2), or the development of lacunae (Figure 6.4a). These effects were particularly apparent when HBSS with 10mM EDTA or IMDM with 5mM RGDS were used as media during blockading experiments (Section 6.5.1), and indeed on occasion RGDS caused complete detachment of the stroma from the plastic carrier. IMDM with 5mM RGEs did not disrupt the stromal layer. The use of several substrates to augment stromal adhesion was explored. Poly-L-Lysine was found to be particularly toxic to cell growth, even after thorough washing of the plate with culture medium. Coating of the plates with collagen under standard conditions (Chapter 4.3) prior to introduction of the cells was found to improve the stability of the M210-B4 stroma [Freshney, 1987] and to prevent disruption of the stroma during divalent cation chelation or exposure to RGDS. Pre-coating with collagen type IV was used as standard protocol in view of the low binding affinity of haematopoietic cell lines for this protein under normal conditions (such that it could be considered non-contributory to binding to the stromal substrate) (Tables 5.3 - 5.7).

**Figure 6.2 Disruption of M210-B4 Stroma.**



Legend. Disruption of M210-B4 cell line with rolling-back of the intact fibroblast layer. (magnification x4).

### 6.2.3 Preparation of human fibroblast and stromal cultures.

The cellularity of a stromal culture is affected by several factors: the exposure of the donor to prior chemotherapy, the volume of the aspirate, and the method of erythrocyte separation. Bone marrow derived from clinical samples was found to be unsatisfactory for stromal culture because of the small volumes available and limited proliferation due to prior exposure to chemotherapy. Normal haematopoietic bone marrow was derived from the posterior iliac crests of patients undergoing general anaesthesia for an elective (non-malignant) surgical procedure, or from volunteers under local anaesthesia. A maximum of 10-20ml was aspirated specifically for culture purposes under sterile conditions. Larger volume aspirates lead to greater dilution of the stromal elements by the haematopoietic elements, which is undesirable in this context. Unless removed, erythrocyte contamination of unseparated cultures leads to a persistent mass over the surface of the culture. Several methods were explored to remove erythrocytes including gravity sedimentation over 30min through 10ml of 1% Dextran [David Kilpatrick - personal communication], or 10ml of 0.01% methylcellulose [Elaine Spooncer - personal communication]. These approaches proved unsatisfactory due to high residual erythrocyte contamination. Bone marrow samples were therefore fractionated by discontinuous density centrifugation over Ficoll-Hypaque at 500g for 30 minutes, and washed several times in sterile HBSS to remove residual Ficoll which may be toxic. This was found to be the most effective approach for removing erythrocytes, although the extent to which stromal elements are lost is unclear. The cells were resuspended in culture medium at a concentration of  $2 \times 10^6$  cells/ml, and 0.5ml aliquots distributed to individual wells of the 24 well plate [Freshney, Pragnell & Freshney, 1994].

Two forms of culture technique were used for bone marrow stromal culture (LTBMC) [Dexter, Spooncer, Simmons *et al.* 1984]. The first used standard culture medium (Appendix 2) and was carried out at 37°C, 5% CO<sub>2</sub> and 100% humidity - conditions known to favour fibroblast outgrowth (Figure 6.3). The second used specific LTBMC medium containing hydrocortisone and horse serum (Appendix 2),

and was carried out at 5% CO<sub>2</sub> and 100% humidity for 3-4 days at 37°C, followed by prolonged incubation at 33°C. The latter conditions have been found to favour adipocyte formation and haematopoiesis (Figure 6.4) as discussed above (Section 6.2.1). Antifungal agents were not used in view of their toxicity to the cultured cells.

All preparative manipulation was carried out under aseptic conditions using a laminar flow hood and sterile glassware or plastic passivated with 1% dBSA in PBS to minimise the loss of adherent cells during cell preparation and manipulation. Bone marrow was manipulated and plated as soon as possible after harvesting, and stored at +4°C if delay was unavoidable. 50% of the supernatant was removed and replaced at weekly intervals, and the cultures monitored by inverted microscopy. It was found that the stromal layers were fragile, and easily disrupted by enthusiastic medium change (Figures 6.2 & 6.4a), in much the same way as the M210-B4 cell line. Precoating the wells with collagen IV was found to provide more stability to the stromal layer. Precoating culture plates with ECM has been reported to augment the rate of stromal development [Campbell, Long & Wicha, 1987], though this was not observed in this work.

### **6.3 Preparatory Experiments to Study the Adhesion of KG1a to *in vitro* Tissue Cultures.**

<sup>51</sup>Cr-labelling of KG1a and substrate adhesion was carried out using the standard protocol (Chapter 4 and Table 4.20). Two concerns arose which were specific to the study of cellular substrates: that removal of the supernatant and washing may lead to disruption of the substrate (discussed in Section 6.2.1 and 6.2.2) and that free <sup>51</sup>Cr present in the supernatant may be taken up by the cellular substrate leading to a falsely high adherent count. Two modifications were therefore made to the standard protocol. All wells were examined by microscopy at the outset and again following supernatant removal and washing at the end of the distractive phase of the experiment. In practice very little disruption of the adherent layer could be ascribed to the washing procedures, especially with the use of collagen as a base substrate. It is possible that a collagenous substrate may alter the structure of the culture in such

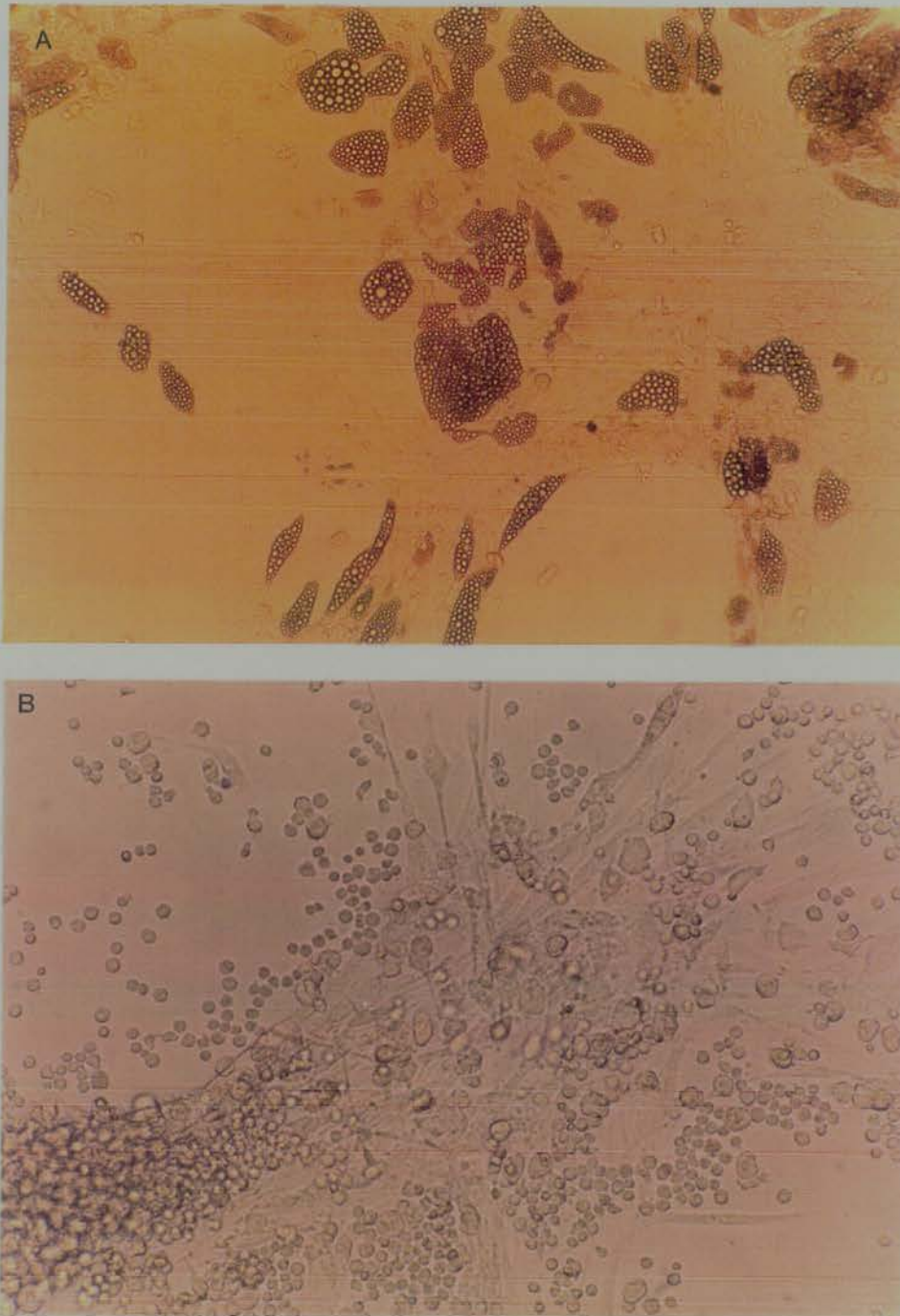


**Figure 6.3 Human Bone Marrow Fibroblast Cultures.**



*Legend.* Human bone marrow fibroblasts grown to confluence in standard tissue culture medium. (magnification x10 blue filter).

**Figure 6.4 Human Bone Marrow Stromal Cultures.**



*Legend.* Human bone marrow stroma grown to confluence in long-term culture medium. The stromal culture differs from a fibroblast culture in the formation of adipocytes (A) (magnification x4) and marked proliferation of primitive HPC to form "cobblestone areas"(bottom left) (B) (magnification x20 blue filter).



a way as to change adhesive interactions with HPC. No difference was apparent in the gross structure of the culture on microscopy. In a further series of experiments a triplicate series of control wells was established containing M210-B4, but exposed only to the appropriate volume of supernatant and not to labelled cells. The adherent and non-adherent counts were analysed in the usual fashion. It was found that counts of the adherent layer were not significantly above background and represented <0.5% of the cell count per well, suggesting that  $^{51}\text{Cr}$  uptake by the substrate was negligible (Table 6.1).

An important further consideration in using stromal LTBM as a substrate for HPC adhesion experiments is whether endogenous haematopoietic activity can (or should) be removed. It could be argued, for example, that autologous HPC may occupy available microenvironmental niches and reduce stromal adhesion of the test cells. It is theoretically possible to grow a stroma free of endogenous haematopoiesis by sorting fibroblast progenitors on the basis of CD34 and Stro-1 positivity. Simmons and Torok-Storb [1991b] have shown that a purified population of CD34<sup>+</sup> STRO-1<sup>+</sup> cells contains >95% of detectable CFU-F, and gives rise to a complex mixture of adherent stromal cells in culture, including fibroblasts, adipocytes, and smooth muscle cells, but not endothelial cells or monocyte-macrophages. Commoner methods of eliminating endogenous haematopoiesis include early or late passage, or irradiation of the stroma following confluence [Moreau, Andreoni, Caux *et al.* 1992]. Stroma treated in this way remains capable of supporting exogenous HPC, but the effects on the stromal microenvironment are uncertain. In a series of experiments, KG1a adhesion to stromal LTBM and fibroblast LTBM with and without collagen type IV as a substrate, were compared directly with adhesion to M210-B4 (Tables 6.2 & 6.3). No significant differences could be detected in KG1a adhesion to stromal and fibroblast layers, and no effect of collagen type IV could be demonstrated. Adhesion to human LTBM was however superior to that to M210-B4, and adherence to all cellular substrates was markedly superior to that to pFn. It appears therefore that KG1a adherence to stroma is superior to that to purified ECM components suggesting that cellular binding

**Table 6.1  $^{51}\text{Cr}$  Uptake by the Adherent Stromal Layer.**

Cells per well:  $4 \pm 1.6 \times 10^5$

Counts per well:  $10,974 \pm 2,632$  cpm

Counts per well (supernatant only):  $234 \pm 163$  cpm

Supernatant contamination:  $2.3 \pm 2.0\%$

M210-B4 layer:  $44 \pm 4$  cpm

Blank:  $40 \pm 2$  cpm

*Legend.* Stromal counts were not found to be significantly above those of blank assays ( $p=\text{ns}$ : paired  $t$  test), and represented  $<0.5\%$  of the total cell count in each well.

**Table 6.2 Comparative Adhesion of KG1a to the M210-B4 Cell Line and to Human Bone Marrow Fibroblast and Stromal Cultures.**

	<i>mean ± SD</i>	<i>cf:dBSA*</i>	<i>cf:pFn<sup>s</sup></i>	<i>(n)</i>
M210-B4	76 ± 8%	p<0.01	p<0.02	4
LTBMC-Stroma (plastic)	92 ± 3%	p<0.001	p<0.01	4
LTBMC-Fibroblast (plastic)	92 ± 3%	p<0.001	p<0.01	4
LTBMC-Stroma (collagen)	91 ± 4%	p<0.01	p<0.02	3
LTBMC-Fibroblast (collagen)	92 ± 1	p<0.01	p<0.02	3
pFn	39 ± 9	p<0.02		4
dBSA	17 ± 8			4

*Legend.* Comparative adhesion of KG1a to cellular substrates. Results presented as the mean ± standard deviation of 3 or 4 replicate experiments. Student's paired *t* test was used to compare results of fibroblast / stromal adhesion to dBSA\* and pFn<sup>s</sup> adhesion (*vide supra*) and between different cellular substrates (Table 6.3). Substantial adhesion occurred to all cellular substrates. Adhesion to human fibroblast and stromal layers was superior to that to M210-B4. There was little difference in KG1a adhesion to fibroblast and stromal with or with out collagen precoating of the plastic.

**Table 6.3 Statistical Comparison of KG1a Adhesion to Various Combinations of Cellular Substrate.**

fibroblast: stroma	ns
fibroblast (collagen): stroma (collagen)	ns
fibroblast: fibroblast (collagen)	ns
stroma: stroma (collagen)	p<0.05
fibroblast: M210-B4	p<0.05
stroma: M210-B4	p<0.05
fibroblast (collagen): M210-B4	p<0.05
stroma (collagen): M210-B4	p<0.05

*Legend.* see Table 6.2.

affinity is greater, perhaps due to the involvement of multiple adhesion pathways. In addition, ongoing endogenous haematopoiesis does not seem to interfere with KG1a adherence.

**6.4 Comparative Adhesion of Haematopoietic Cell Lines to M210-B4.**

A comparative study of the adhesion of the haematopoietic cell lines KG1a, K562, HL60, CEM and NALM-6 to M210-B4 was carried out to establish whether differences in CAM expression and adhesion to ECM components translated into differences in adhesion to composite stromal layers. The results of these experiments are summarised in Table 6.4. All the haematopoietic cell lines studied adhered to M210-B4, though the lymphoblastic lines CEM and NALM-6 demonstrated significantly greater stromal adherence than KG1a.

**Table 6.4 Comparative Adhesion of Haematopoietic Cell Lines to M210-B4.**

<i>Cell line.</i>	<i>mean ± SD</i>	<i>cf: KG1a</i>
KG1a	64.3 ± 4.2%	
K562	62.9 ± 1.3%	ns
HL60	72.4 ± 13.5%	ns
CEM	81.4 ± 2.0%	p<0.01
NALM-6	80.3 ± 2.4%	p<0.02

*Legend.* Data presented as the mean ± standard deviation of 4 replicate experiments. Adhesion to M210-B4 was compared to that of KG1a by non-paired *t* test. All cell lines adhered to M210-B4, though the lymphoid cell lines NALM-6 and CEM demonstrated significantly greater adhesion than KG1a.

## **6.5 Blockade of KG1a Adhesion to M210-B4.**

### *6.5.1 Blockade of KG1a adhesion by divalent cation chelation and synthetic peptides.*

Experiments were carried out to study the adhesion of  $^{51}\text{Cr}$ -labelled KG1a to M210-B4 in the presence of HBSS 10mM EDTA, 5mM RGDS or RGES peptides. KG1a were incubated with  $^{51}\text{Cr}$  and peptide as described (Chapter 5.4.4). As noted previously the adherent layer was found to become completely distracted from the plastic carrier in the presence of divalent cation chelation and RGDS peptides, suggesting that the stromal layer secretes its own ECM to which it then adheres through an RGD-dependent mechanism. Precoating the wells with collagen type IV protected the stroma from disruption presumably through the involvement of non-integrin-dependent adhesion mechanisms. The results are summarised in Table 6.5. Divalent cation chelation significantly blocked KG1a adhesion to M210-B4, but 5mM RGDS caused no significant blockade, and indeed there was no significant difference between adhesion in the presence of RGDS compared to that in the presence of RGES ( $p=\text{ns}$  paired  $t$  test).

### *6.5.2 Blockade of KG1a adhesion by monoclonal antibodies.*

Experiments were carried out to study the effects of mAbs known to blockade adhesion in other experimental systems. LB-2 (anti-ICAM-1 CD54) has been shown to inhibit ICAM-1 mediated adhesion to LFA-1 [Makgoba, Sanders, Luce *et al*, 1988]. L133.1 (anti-PECAM-1 CD31) has been shown to inhibit homotypic-mediated aggregation of PECAM-1 expressing L cells [Albelda, Muller, Buck *et al*, 1991]. G25.2 (anti-LFA-1 $\alpha$  CD11a) has been shown to inhibit LFA-1:ICAM-1 interaction [Makgoba, Sanders, Luce *et al*, 1988], whilst L178 (anti-HCAM CD44) has been shown to inhibit lymphocyte adhesion to lymph node endothelial cells [Jalkanen, Bargatze, de los Toyos *et al*, 1987]. L25.3 (anti-VLA-4 $\alpha$  CD49d) and mAb 16 (anti-VLA-5 $\alpha$  CD49e) were also used as previously noted (Chapter 5.4.5). Three adhesion molecules were not studied. It was thought unlikely that LFA-3 (a counter-receptor for T lymphocyte expressed LFA-2) would play a major role in



**Table 6.5 Blockade of KG1a-M210-B4 Adhesion by Divalent Cation Chelation and RGDS Peptides.**

<i>medium</i>	<i>mean ± SD</i>	<i>cf: dBSA*</i>	<i>cf: no blockade<sup>s</sup></i>
IMDM + 10% FCS	73.1 ± 12.5%	p<0.01	
HBSS + 10mM EDTA	42.1 ± 10.8%	p<0.05	p<0.05
IMDM + 5mM RGDS	65.6 ± 8.0%	p<0.01	ns
IMDM + 5mM RGES	61.0 ± 10.2%	p<0.001	ns
dBSA	14.3 ± 8.7%		

*Legend.* Data is presented as the mean ± standard deviation of 4 replicate experiments.

Statistical comparisons to dBSA\* and to M210-B4 without blockade<sup>s</sup> by paired *t* test. Divalent cation chelation blocked KG1a adhesion to M210-B4, but 5mM RGDS or RGES had no effect. There was no significant difference between adhesion in the presence of RGDS or RGES (p=ns).

KG1a adhesion to M210-B4 and so blockading studies with mAbs to this CAM were not carried out. Blockading mAbs to CD36 were not available at the time of the study whilst L-Selectin is not expressed by KG1a (Figure 3.8 - 3.10). mAbs were titrated against KG1a using indirect immunofluorescence as previously described (Chapter 5.4.5) and were used at a final concentration of  $1\mu\text{g} / 5 \times 10^5$  cells. The results of these experiments are summarised in Table 6.6. None of the mAbs studied proved capable of blockading KG1a adhesion to M210-B4.

#### *6.5.3 Blockade of KG1a adhesion by enzymatic treatment.*

A final group of experiments was carried out to study the effects of glycolytic enzymes on KG1a adhesion to M210-B4.  $^{51}\text{Cr}$ -labelled KG1a were incubated with chondroitinase ABC, heparinase I or hyaluronidase prior to the adhesive phase of the experiment as previously described (Chapter 5.4.6). The results are summarised in Table 6.7. Once again no significant blockade of KG1a adhesion to stroma could be demonstrated.

#### *6.5.4 Discussion.*

The series of experiments described in the preceding sections demonstrated that the  $^{51}\text{Cr}$  adhesion assay is applicable to the study of haematopoietic cell line adhesion to complex tissue culture layers *in vitro*. Unlike the situation where cell line adhesion to ECM components was studied (Chapter 5), it proved impractical to blockade KG1a adhesion to stroma with selective blocking agents (synthetic peptides, mAbs or enzymes). The relatively non-specific approach of divalent cation chelation was partially effective, though stromal adhesion remained greater than that to background (dBSA). This data suggests that multiple adhesive pathways (some of them divalent cation dependent) are involved, leading to a degree of redundancy in HPC-stromal adhesion.

It was clear that the application of the  $^{51}\text{Cr}$  adhesion assay to HPC required the development of high-grade enrichment and  $^{51}\text{Cr}$ -labelling of the target population.

**Table 6.6 Blockade of KG1a-M210-B4 Adhesion by Monoclonal Antibodies.**

<i>mAb</i>	<i>mean ± SE</i>	<i>cf: dBSA*</i>	<i>cf: no mAb<sup>s</sup></i>
nil	73.1 ± 12.5%	P<0.005	
ICAM-1	68.7 ± 12.9%	P<0.005	ns
PECAM-1	76.9 ± 9.4%	P<0.005	ns
LFA-1	77.9 ± 11.2%	P<0.005	ns
HCAM	77.1 ± 13.5%	P<0.005	ns
VLA-4	58.8 ± 28.7%	P<0.05	ns
VLA-5	62.7 ± 26.8%	p<0.05	ns
IgG1 (control)	69.1 ± 13.1%	P<0.01	ns
dBSA	15.9 ± 11.6%		

*Legend.* Data is presented as the mean ± standard error of 4 replicate experiments. KG1a adhesion to M210-B4 in the presence of the named mAbs is compared to that in the absence of mAb<sup>s</sup> or to dBSA\* by paired *t* test. None of the mAbs tested showed demonstrable effect on adhesion.

**Table 6.7 Blockade of KG1a-M210-B4 Adhesion by Enzymes.**

<i>enzyme</i>	<i>mean ± SD</i>	<i>cf: dBSA*</i>	<i>cf: nil<sup>s</sup></i>
nil	79.0 ± 11.9%	p<0.01	
Chondroitinase ABC	77.9 ± 16.9%	p<0.01	ns
Heparinase I	75.1 ± 18.6%	p<0.05	ns
Hyaluronidase	73.7 ± 14.7%	p<0.02	ns
dBSA	24.3 ± 11.0%		

*Legend.* Data is presented as the mean ± standard deviation of 4 replicate experiments. KG1a adhesion to M210-B4 in the presence of the named enzymes is compared to that in the absence of enzyme<sup>s</sup> or to dBSA\* by paired *t* test. None of the enzymes tested showed demonstrable effect on adhesion.

## **6.6 Development of a Haematopoietic Progenitor Enrichment Protocol.**

### *6.6.1 Background and definitions.*

Several approaches can be taken to HPC enrichment, and these are outlined in Table 6.8 [Visser & Van Bekkum, 1990; Wunder & de Wynter, 1994]. Physical methods exploit differences in cell size (elutriation) or density (sedimentation, density gradient centrifugation). These are often used as a preparatory step in order to effect depletion of red cells and / or neutrophils. The effects of Ficoll-Hypaque separation on HPC enrichment and loss were explored in Section 6.6.3.

A number of different approaches can be taken to negative selection. Initially workers in the area utilised attributes of mature cells such as rapid monocyte adherence to plastic or T lymphocyte rosette formation with sheep erythrocytes, to enrich for HPC. To avoid multiple sequential depletion steps, naturally occurring lectins such as soybean agglutinin were used to deplete a broad spectrum of mature cells. Latterly, it has become feasible to remove mature cells on the basis of a panel of mAbs, associated with bead, column or complement-mediated effectors [Bertoncello, Bradley & Watt, 1991]. The problems with approaches based on negative selection relate to poor enrichment, particularly when the target population comprises only a small fraction of the initial population.

The CD34 antigen can be used along with a number of different effector systems to provide a single parameter positive selection. Briefly, these can be categorised into those in which the CD34 mAb is covalently attached to the surface of a plastic flask (AIS system), those in which biotinylated CD34 mAbs adhere to a streptavidin column (Cell-Pro), and those in which CD34 mAbs are linked to beads and removed using a magnetic field (Dynal, Baxter, Miltenyi Biotec) (Table 6.8). These approaches have relative advantages and disadvantages, but an important problem is the nature of the residual label left on the surface of the cell. For example, the Class II anti-CD34 mAb QBEND10, but not the Class III mAb 8G12, has recently been shown to enhance adhesiveness through LFA-1 activation [Majdic, Stöckl, Pickl *et al*, 1994; Traore & Hirn, 1994]. The cumulative effect of mAbs and beads on the surface of a cell is unknown. Manufacturers of these systems claim CD34

**Table 6.8 Haematopoietic Progenitor Enrichment.**

*Physical methods*

Sedimentation (eg. methylcellulose, dextran)

Density gradient centrifugation (eg. Ficoll, Percoll)

Centrifugal counterflow elutriation (eg. CCE, Beckman).

*Negative immunological selection.*

*Single parameter positive immunological selection.*

Immune panning (eg. AIS)

Immunoabsorption columns (eg. CellPro)

Immunomagnetic beads

Large beads (near cell size) (eg. Dynal, Baxter)

Medium beads (eg. Immunotech)

Small beads (eg. Miltenyi Biotech)

*Multiparameter positive immunological selection.*

Fluorescence activated cell sorting.

---

enrichment of up to 98%, but in general laboratory practice a figure of around 30-50% has proved more realistic amongst European research laboratories. Indeed in a European workshop partially devoted to HPC enrichment, manufacturers were unable to replicate the levels of enrichment claimed in their own laboratories [Silvestri, Wunder, Sovalat *et al.* 1993; Wunder, Sovalat, Hénon *et al.* 1994]. The advantage of a single step immunoabsorption or immunomagnetic enrichment method lies in the processing of large numbers of cells albeit to an intermediate purity [Winslow, Liesveld, Ryan *et al.* 1994].

It was necessary to consider a third option to provide highly enriched (>98%) CD34<sup>+</sup> HPC populations, based on the multiparameter sorting capabilities of the fluorescence activated cell sorter (FACSorter). This instrument operates on the same principles as the FACScan (Chapter 2.4.1). Subpopulations of cells from a heterogeneous population are identified on the basis of light scatter and fluorescence



characteristics [Parks, Lanier & Herzenberg, 1986]. The nozzle assembly of the sorter is vibrated by an oscillator-driven piezoelectric crystal at a frequency close to the natural drop break up frequency of the jet. This stabilises drop formation at that frequency, resulting in a uniform drop size, and a well defined time delay between characterisation of the cell, and incorporation in the free drop. If the cell is to be sorted, a voltage of approximately 100 volts is applied to the fluid just inside the nozzle, and a drop which breaks from the jet whilst the voltage is applied will carry the corresponding charge. The train of drops passes between two deflection plates charged at +/- several thousand volts, and the charged drops are deflected from the uncharged, and collected separately. Sorting decisions are made by combining acquisition gates on the signals, so that a cell is sorted if its light scatter properties and each of its fluorescence signals falls within defined limits. Large numbers of cells may need to be examined in order to sort a rare subpopulation, and multiple parameters may have to be utilised.

The same generic problem occurs when sorting HPC on the basis of CD34<sup>+</sup> fluorescence as has already been discussed in some depth *a propos* the definition of the study population in two and three colour immunocytometry (Chapter 2.9.3 & 3.5) [Weichel, Irlenbusch, Kato *et al.* 1992]. The position of the discriminatory marker is a trade off between the selectivity of the sorting process in favour of the more primitive CD34<sup>hi</sup> population, and the extent of contamination of the study population with false positive cells. Enrichment of a rare cell subpopulation by immunomagnetic and immunocytometry will amplify the false positive population *pro rata* with the true positive. The approach taken to FACSorting a CD34<sup>+</sup> population was similar to that employed during three colour and light scatter analysis (Chapter 3.5), namely that negative and positive controls were used to establish an acquisition gate on the FSC / SSC dot-plot, and a negative control population was used to establish a second acquisition gate on FL-2 at 0.1% false positivity (nested acquisition gates can be established on the FACSsorter) and to calculate the percentage contamination of the acquired CD34<sup>+</sup> positive population. This data was used to correct the measured CD34%<sup>+</sup> of the tertiary product to get a

more accurate estimate of the true purity of the study population. An alternative quality control over the enrichment process would have been to counterstain the final product with an anti-CD34- FITC conjugate directed to a different epitope (*e.g.* QBEND10-FITC).

In a sequential enrichment protocol such as this, the product of one enrichment process acts as the source material for the next. Some clarification of the nomenclature is required to avoid confusion. The starting material (*e.g.* bone marrow, peripheral blood or cord blood) is referred to as the *initiate*. The product of a density gradient centrifugation (mononuclear cells) or of leucapheresis (nucleated cells) is termed the *primary product*, the product of an immunoadsorbitive step is termed the *secondary product*, and the product of a FACSsort is termed the *tertiary or final product*. Terms are maintained even if one or more steps are omitted *e.g.* FACSsorted leucapheresis cells are still referred to as a *tertiary product*.

The enrichment protocol was evaluated by CD34 immunocytometry, cell enumeration (by automated counter or haemocytometer) and viability by trypan blue dye exclusion (haemocytometer) (Chapters 2.2.3 and 2.4).

The following derivative terms were defined:

*enrichment:*     
$$\frac{\% \text{ CD34 positive cells in the product}}{\% \text{ CD34 positive cells in the initiate}}$$

*% recovery:*

$$\frac{(\% \text{ CD34 positive cells in the product}) \times (\text{total number of cells in product})}{(\% \text{ CD34 positive cells in the initiate}) \times (\text{total number of cells in initiate})} \times 100.$$

*purity:*             
$$\% \text{ CD34}^+ \text{ of the product} - \% \text{ contamination of CD34}^+ \text{ population in the initiate}$$

### *6.6.2 Preliminary experimental studies.*

In a preliminary experiment,  $10^8$  cells from leucapheresis product were labelled with 100 $\mu$ l of 8G12-RPE using standard single colour staining technique. The cells were sorted using nested acquisition gates established on FSC / SSC and on FL2 as described above, to include <0.1% of the negative control population. The results are summarised in Table 6.9. This preliminary experiment was sufficient to establish that the staining procedure, purity and recovery were satisfactory, but that the sorting time for populations of this kind was not. The FACSsorter has a maximum sort rate of 5,000 cells/sec, equating to theoretical sort of 75 CD34<sup>+</sup> cells/sec or  $1 \times 10^6$  cells in 4hrs for this sample. In practice the CD34<sup>+</sup> sort rate was 10-20 cells/sec, yielding  $1 \times 10^6$  CD34<sup>+</sup> cells in 14hrs. This was not considered a practical option, and clearly some form of pre-sort enrichment was necessary.

It was decided that Ficoll-Hypaque discontinuous density centrifugation would be used to prepare mononuclear cells from PB or BM (but not LP) because red cell contamination was found to swamp both the immunomagnetic selection and the FACSsorter. This was followed by a preparatory positive immunological selection and a final high grade enrichment by FACSsorter.

### *6.6.3 Effects of Ficoll-Hypaque gradient on CD34 quantitation.*

The effects of discontinuous density gradient centrifugation on CD34 quantitation was examined in a series of four experiments. 4ml of Ficoll (density 1.077g/dl) was decanted into a 14ml test tube and 10ml of bone marrow was added gently onto the top using a Pasteur pipette such as to preserve the integrity of the Ficoll-BM interface. The samples were subject to centrifugation at 500g for 30min. The mononuclear cell layer was removed carefully by pipette and the cells washed twice in HBSS. Samples of BM prior to and following separation were immunofluorescence labelled and subject to red cell lysis in the usual manner (Chapters 2.4). Both samples were treated with red cell lysis to maintain comparability. The results are summarised in Table 6.10. 15% of the leucocytes from the initial sample were recovered from the Ficoll gradient, with a CD34%<sup>c</sup>

**Table 6.9 Pilot Cell-Sort of 10<sup>8</sup> Cells from Leucapheresis Product.**

Initial CD34<sup>+</sup> count: 1.52% (5x10<sup>5</sup> cells labelled).

Initial cell count: 1x10<sup>8</sup> cells

Initial CD34<sup>+</sup> count on primary product: 1.59%

CD34<sup>+</sup> count through the lymphoblastoid gate: 2.50%

Cell recovery in tertiary product: 1.5x10<sup>6</sup> cells

CD34<sup>+</sup> purity in the tertiary product: 95%

Enrichment: 59.7 fold                  Recovery: 98.7%

---

**Table 6.10 Results of Preparatory Separation of Bone Marrow Samples over Ficoll-Hypaque.**

	<i>total leucocyte count</i>	<i>CD34%<sup>c</sup></i>	<i>CD34<sup>n</sup> count</i>
pre-separation	221 ± 102x10 <sup>6</sup>	1.25 ± 0.62	3.24 ± 2.43x10 <sup>6</sup>
post-separation	38 ± 24x10 <sup>6</sup>	6.11 ± 2.94	2.57 ± 2.43x10 <sup>6</sup>

*Legend.* Purity 6.1 ± 2.9%. Enrichment: 4.88 ± 0.50 fold. Recovery: 77 ± 25%.

purity of 6.11%, a mean CD34 enrichment of 4.88 fold and a mean CD34 recovery of 77.25%.

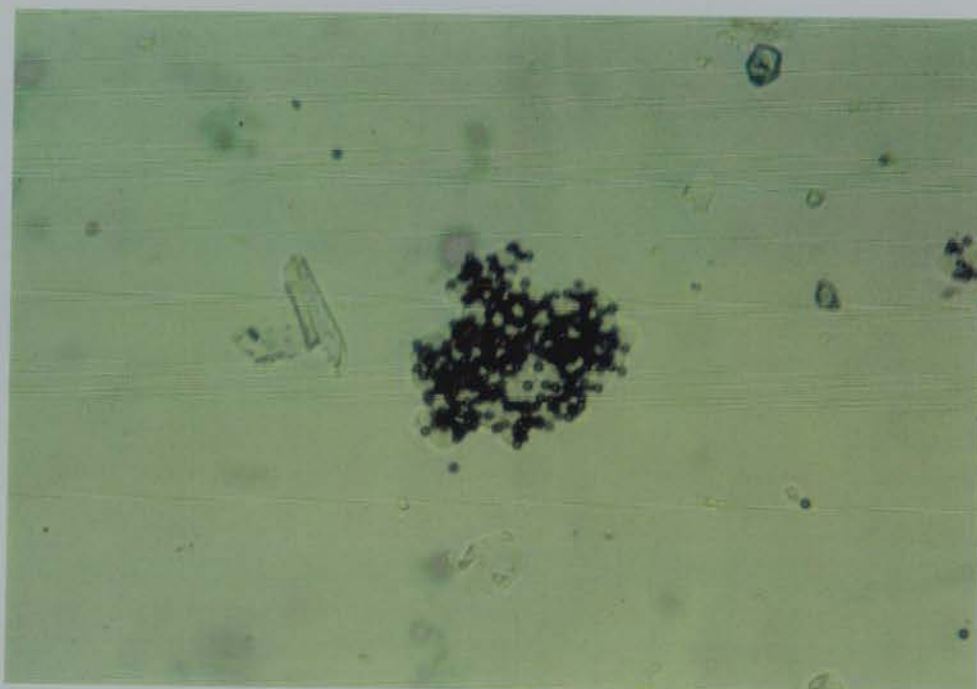
#### *6.6.4 The use of Dynal anti-CD34 beads and bead detachment systems.*

The use of Dynal beads as a method for positive selection of HPC was investigated. Cells were labelled with an anti-CD34 mAb 561 directly conjugated to a Dynal bead [Egeland, Tjonnfjord, Steen *et al.* 1993; Egeland & Gaudernack, 1994]. The bead diameter is  $>0.5\mu\text{m}$  and confers a relatively large magnetic moment to the labelled cell, which can be easily separated from the unlabelled population using a simple permanent magnet. Although this system was found to be simple and cheap, several drawbacks limited its applicability as a preparatory enrichment for FACSorting. Multiple cell-bead attachments occurred leading to rosetting of and aggregation of the CD34<sup>+</sup> cells with beads (Figure 6.5). In addition, the beads were found to interfere with FACS analysis by changing the optical and fluorescence properties of the cells (Figure 6.6). Finally, it seemed likely that the beads may interfere with adhesive properties of the study population. It was clear, therefore, that if the Dynal system were to be of value the beads would have to be removed from the surface of the cells prior to FACSorting. Several groups have reported that incubation of cells overnight would lead to spontaneous shedding of the beads. Trial experiments demonstrated very little bead shedding from KG1a using this method.

Civin reported that populations of 50-90% purity, 40-60% recovery and 100% viability could be achieved using My10 and SAM- immunomagnetic microbeads, shed by incubation with chymopapain at a concentration of 200u/10<sup>7</sup> cells/ml for 10min at 37°C [Civin, Strauss, Fackler *et al.* 1990]. Although there was little effect on the morphologic and light scatter characteristics of the enriched cells, selective loss of CD34 epitopes was reported. This was confirmed in an experiment in which chymopapain treated KG1A were found to stain with 8G12-RPE but not with QBEND10-FITC demonstrating that the Class II but not the Class III epitopes of CD34 are chymopapain-sensitive. Clearly, therefore, this is an unsuitable approach



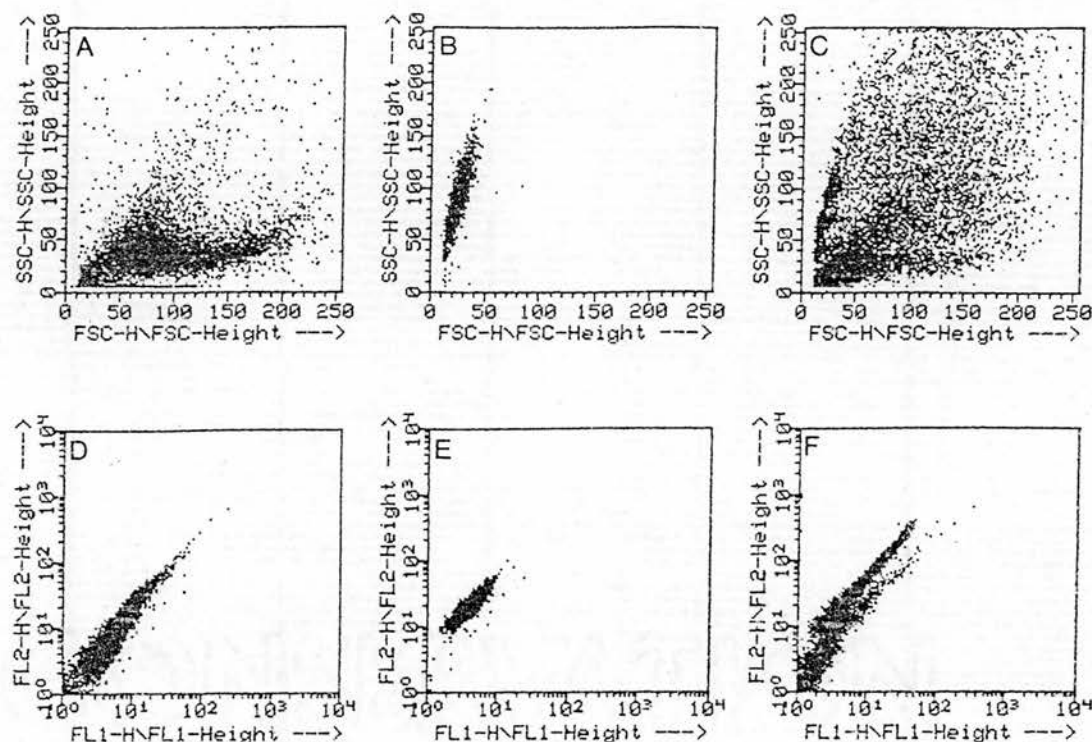
**Figure 6.5 KG1a Labelled with Dynal Beads.**



*Legend.* KG1a-labelled with Dynal anti-CD34 beads. The beads are easily visible by microscopy and lead to rosetting and aggregation of the CD34<sup>+</sup> population. (magnification x20).



Figure 6.6 Physical and Fluorescence Characteristics of KG1a Labelled with Dynal Beads.



*Legend.* The FSC / SSC characteristics of unlabelled KG1a (A) and Dynal beads (B) have a combined effect when KG1a are labelled with the latter (C). Moreover, the FL-1 / FL-2 characteristics of KG1a (D) differ from those of the beads (E) giving rise to a complex fluorescence population on the labelled sample (F). These properties make Dynal beads somewhat unsatisfactory when used for fluorescence enrichment or analysis of a target population.

for detailed studies of membrane antigen expression and function.

Egeland *et al.* have carried out a considerable amount of work in the development of the Dynal system and have been able to detach the beads from the target population using a polyclonal Rabbit anti-mouse serum (detachabead) [Smeland, Funderud, Kvalheim *et al.* 1992]. A series of experiments was carried out in collaboration with Janet Innes of the Scottish National Blood Transfusion Service National Sciences Laboratory to investigate the efficacy of the Dynal detachabead system. KG1a were incubated with 561-Dynal beads at a ratio of 4 beads / cell, for 1 hour at ambient temperature. The number of cells present and the percentage rosetted with beads was enumerated using a haemocytometer. The cells were washed several times to remove unrosetted cells, by placing the tube within the magnetic field, extracting the supernatant by pipette, removing the tube from the field and resuspending the cells in handling medium. The rosetted cells were enumerated and incubated in 10µl of detachabead /  $10^6$  beads for 1 hour at ambient temperature. Unrosetted cells were removed from the beads and residual rosetted cells using the same washing procedure as previously, and both populations were enumerated. The results of these experiments is summarised in Table 6.11. More than 90% of KG1a formed rosettes, comparable to the percentage of this population which express CD34 (Chapter 2.4.6 and Figure 2.6). Approximately 30% were recovered following bead detachment, with another 20% remaining rosetted. In two experiments the rosetted cells were subject to a second incubation with detachabead leading to recovery of a further 16% of the initial population with 5% of the initial population remaining rosetted. Two problems were considered to be of particular importance. First, up to 50% of the cells were lost from the system, suggesting traumatic damage during the washing procedures. Second, the partition between rosetted and non-rosetted cells by the magnet was not complete. In particular the washed population following bead detachment still contained cells with single beads attached, which interfered with further FACS analysis or sorting. Furthermore, test experiments with LP and CB showed very little success in isolating the small numbers of CD34<sup>+</sup> cells present in these samples.

**Table 6.11 Results of Experiments Removing Dynal Beads from KG1a using the Detachabead System.**

Cells harvested:  $16 (7-40) \times 10^6$

Rosette formation:  $94 \pm 4\%$

Cells recovered:  $30 \pm 13\%$

Residual rosettes:  $21 \pm 11\%$

---

#### *6.6.5 The use of Miltenyi MACS system.*

The magnetic cell separation system devised by Stefan Miltenyi at Cologne [Miltenyi, Muller, Weichel *et al.* 1990] uses a combination of supramagnetic ferritin-dextran beads with a diameter of approximately 50nm and a high gradient magnetic field to circumvent the small magnetic moment of the beads. The beads are not visible by transmitted-light microscopy. Labelled cells are run through a column containing plastic-coated steelwool, which works as an extremely sensitive filter for magnetic particles when within a magnetic field, but rapidly de-magnetises when removed from the field allowing the retained particles to be eluted. Flow through the column is controlled by a disposable needle at the outlet.

In initial experiments  $10^7$ - $10^8$  cells LP derived cells were labelled with 8G12-RPE and with of a second step rat anti-mouse mAb supramagnetic bead conjugate, using standard indirect immunofluorescence labelling technique (Chapter 2.4, Table 6.12). It was clear that 8G12-RPE could not be used at standard concentration ( $20\mu\text{l} / 10^6$  cells) otherwise 2ml of mAb would have to be used to stain  $100 \times 10^6$  cells. The mAb was therefore re-titrated on a representative LP sample (Figure 6.13). The mfi of the  $\text{CD34}^+$  population declined as expected, but percentage  $\text{CD34}^+$  positivity could be maintained down to a lower concentration of  $1\mu\text{l} / 10^6$  cells by appropriate adjustment of the discriminatory marker, albeit at the cost of increased contamination of the  $\text{CD34}^+$  population. On the strength of this, the 8G12-RPE was used at  $10\mu\text{l} / 10^7$  nucleated cells. This is commensurate with the staining of  $5 \times 10^5$   $\text{CD34}^+$  KG1a with  $10\mu\text{l}$  of 8G12-RPE (Chapter 2.4) because the target  $\text{CD34}^+$

**Table 6.12 Immunofluorescence Labelling Protocol for *MACS* and *FACSort*.**

$10^8$  nucleated cells derived from a leucapheresis product or Ficoll-Hypaque density centrifugation.

washed twice in handling medium

incubated for 15 min in 0.5% gamma globulin

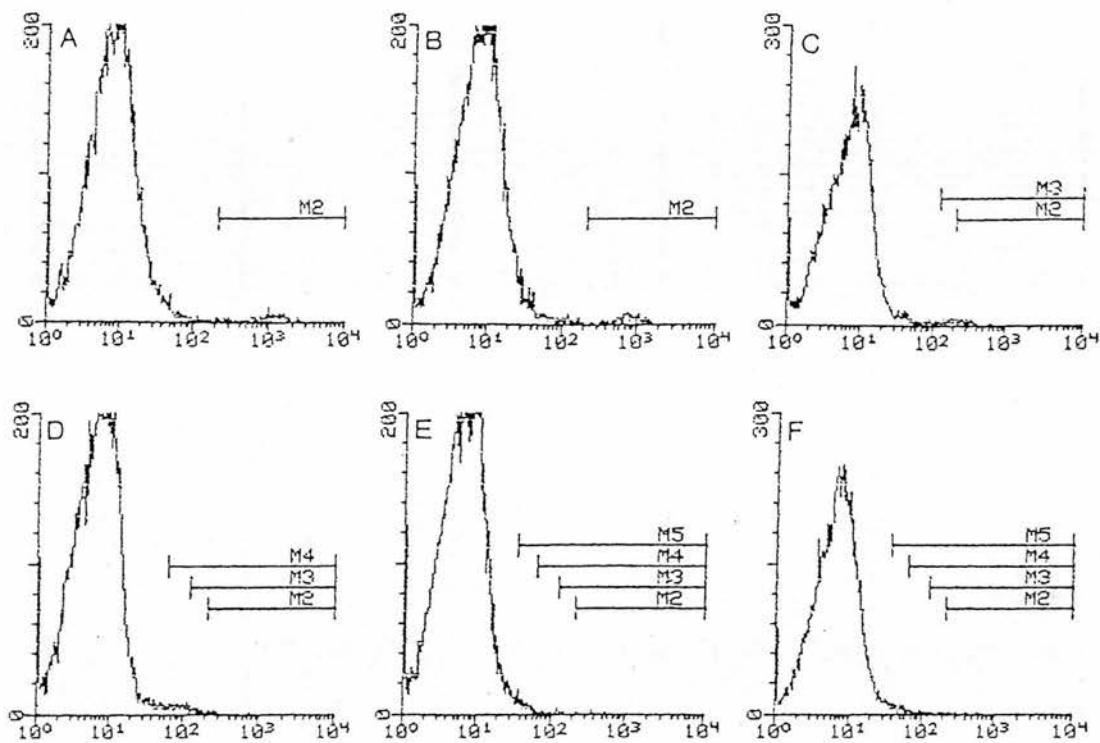
incubated for 15 min with 100 $\mu$ l 8G12-RPE

washed twice in handling medium

incubated for 15 min with 100 $\mu$ l of RAM-microbead conjugate

washed once and resuspended in handling medium.

Figure 6.7 Titration of the anti-CD34 mAb 8G12-RPE on Leucapheresis Product.



*Legend.* Titration of the anti-CD34 mAb 8G12-RPE on  $10^6$  cells from a LP sample revealed, as expected, a progressive fall in the mean fluorescence intensity of the CD34<sup>+</sup> population with reduction in the volume of mAb (A - E). Use of progressively more permissive discriminatory markers on the negative control (F) allowed HPC discrimination down to  $1\mu\text{l} / 10^6$  cells albeit at the cost of greater contamination of the study population with false positive cells. (A)  $20\mu\text{l}$  8G12-RPE %fl<sup>+</sup> (M2) 1.04% mfi 978. (B)  $10\mu\text{l}$  8G12-RPE %fl<sup>+</sup> (M2) 1.15% mfi 750. (C)  $5\mu\text{l}$  8G12-RPE %fl<sup>+</sup> (M3) 1.21% mfi 184. (D)  $1\mu\text{l}$  8G12-RPE %fl<sup>+</sup> (M4) 1.27% mfi 113. (E)  $0.5\mu\text{l}$  8G12-RPE %fl<sup>+</sup> (M5) 1.21% mfi 61. (F) negative control %fl<sup>+</sup> (M2) 0.05% (M3) 0.11% (M4) 0.42% (M5) 1.23%.

population in LP rarely comprises more than 5% of the primary product. An aliquot of the primary product was retained and labelled separately with 8G12-RPE using the standard quantitative methodology (Chapter 2.4) to act as an internal control for the adequacy of the reduced labelling volume, and to aid in satisfactory definition of the CD34<sup>+</sup> population during FACS analysis and sorting. The RAM-microbead conjugate could not be titrated and was used at 20µl / 10<sup>7</sup> cells according to the manufacturers instructions.

*MiniMACS* columns (maximum capacity 10<sup>7</sup> magnetic-labelled cells) were prepared by priming with 500µl of handling medium and discarding the effluent. The column was placed in the magnetic field, a 22 gauge needle attached to the outlet, 2ml of the cell suspension containing a maximum of 10<sup>8</sup> cells was added at the top and the effluent allowed to run through. The needle was removed and a further 2ml of handling medium added and allowed to wash through. The column was removed entirely from the vicinity of the magnetic field, a further 2ml of handling medium added and the retained cells flushed out using the plunger supplied. The eluted cells were enumerated and the CD34<sup>+</sup>% quantitated in the usual manner.

In practice it was found that residual erythrocyte contamination within the LP led to problems in enumeration of the CD34<sup>+</sup> cells because CD45 counterstaining could not be carried out. The primary product was therefore subject to an erythrocyte lysis step following labelling and was then resuspended in handling medium. Inadequate dispersion of the leucocytes in the fluid prior to *MACS* sorting led to blockage of the column. Apart from these preliminary problems the *MACS* system was found to be satisfactory and the results of a series of four experiments carried out on LP are summarised in Table 6.13. The labelled cells were found to have similar physical and fluorescence characteristics to an unlabelled population (Figure 6.8).



**Table 6.13 Results of the Effects of MACS Separation on Leucapheresis Product.**

*Leucapheresis product.*

Cell count:  $82.4 \pm 28.7 \times 10^6$

CD34%<sup>+</sup>:  $1.24 \pm 0.12$

CD34<sup>n</sup>:  $1.03 \pm 0.38 \times 10^6$

*MACS eluate.*

Cell count:  $4.4 \pm 2.4 \times 10^6$

CD34%<sup>+</sup>:  $14.12 \pm 8.1$

CD34<sup>n</sup>:  $0.51 \pm 0.24 \times 10^6$

*Enrichment:*  $11.7 \pm 7.2$  fold. *Recovery:*  $55.1 \pm 26.0\%$ .

---

*6.6.6 Sequential MACS and FACSorting enrichment protocol.*

A series of experiments were carried out in which cells from LP were subject to sequential *MACS* enrichment followed by *FACSorting*. The operating parameters on the *FACSorter* were established and the performance monitored by Andrew Sanderson of the Institute of Cell, Animal and Population Biology, University of Edinburgh. Labelled cells were acquired through an open gate designed to exclude debris and cell aggregates only. CD34<sup>+</sup> cells were discriminated on the basis of a 0.1% marker established on a negative control sample as previously described. CD34<sup>+</sup> cells were enumerated during collection by the *FACSorter*, the percentage positivity in the final product was assessed by passing an aliquot through the *FACScan*, and viability of the final product was assessed by trypan blue dye exclusion. The results of a series of 6 experiments are summarised in Table 6.14. It was not feasible to determine whether selective loss of HPC subsets had occurred, because of the difficulty in obtaining sufficient cells for secondary labelling with lineage and activation markers. It is accepted that the enrichment process favoured the retention of CD34<sup>hi</sup> HPC. Cells from the tertiary product displayed similar light scatter characteristics to those from the primary product (Figure 6.8), and appeared

**Table 6.14 Results of Sequential MACS and FACSort Enrichment of Haematopoietic Progenitors on the Basis of CD34 Positivity.**

*Leucapheresis product.*

Cell count:  $69.2 \pm 24.8 \times 10^6$

CD34<sup>+</sup>:  $1.70 \pm 0.91$

CD34<sup>n</sup>:  $1.18 \pm 0.79$

Contamination:  $4.3 \pm 2.5\%$

*Final product.*

Cell count:  $0.32 \pm 0.35 \times 10^6$

CD34<sup>+</sup>:  $86.6 \pm 10.9\%$

CD34<sup>n</sup>:  $0.25 \pm 0.24 \times 10^6$

*Enrichment:*  $64.6 \pm 32.1$ .      *Recovery:*  $18.8 \pm 8.3\%$       *Purity:*  $82.4 \pm 8.9\%$

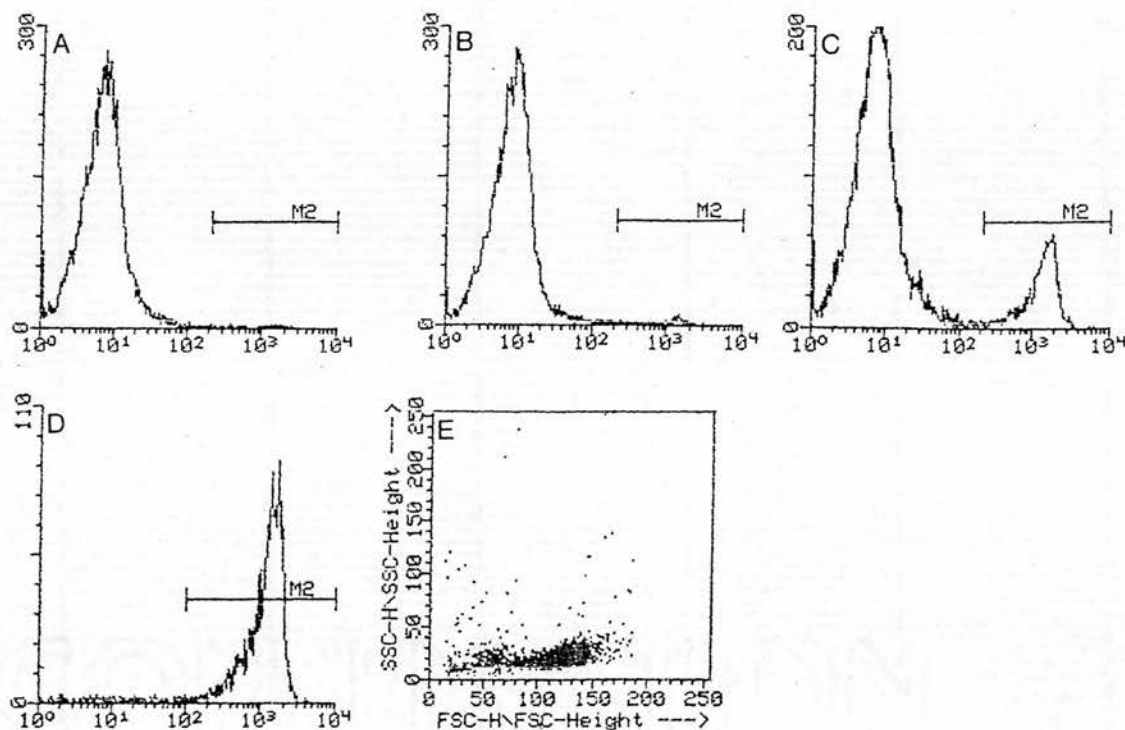
*Viability:*  $>90\%$ .

-----

to be of primitive "blast" morphology when examined by transmitted light microscopy.

In summary, a three-stage protocol was effective in achieving highly enriched populations of HPC, starting with LP or Ficoll-prepared mononuclear cells, followed by a single parameter positive enrichment based on immunomagnetic or immunocolumn technology, and leading to multiparameter selection by FACSorting. The major problems are the high degree of associated cell loss and the potential for alteration in cell function - both of which are currently poorly characterised and may or may not be selective to certain HPC subsets. Since it was clear that only small numbers of HPC would be available for adhesion experiments, the labelling intensity of HPC became a critical issue in considering whether the <sup>51</sup>Cr adhesion assay was applicable to this cell population.

**Figure 6.8 Flow Cytometry of Haematopoietic Progenitors Enriched by *MACS* and FACSsort.**



*Legend.* Representative sequential enrichment of CD34<sup>+</sup> HPC from a leucapheresis product. (A) negative control M2 0.1%. (B) CD34<sup>+</sup> leucapheresis product M2 1.24% (C) *MACS* eluate M2 14.5%. (D) FACSsorted CD34<sup>+</sup> cells M2 98.0%. (E) CD34<sup>+</sup> sorted cells demonstrate fairly typical FSC / SSC characteristics.

## 6.7 Application of the $^{51}\text{Cr}$ Adhesion Radioassay to Highly Enriched HPC.

### 6.7.1 Pilot studies of $^{51}\text{Cr}$ labelling of peripheral blood lymphocytes.

In the first instance the feasibility of increasing the  $^{51}\text{Cr}$  labelling index was assessed using peripheral blood mononuclear cells. These cells were readily available and the minimal preparation meant that the issues related to prolonged *ex vivo* manipulation (preceding Section) could be temporarily ignored. An initial series of experiments was carried out employing the same labelling protocol as for the cell lines (*i.e.* 200kBq  $^{51}\text{Cr}$  in 100 $\mu\text{l}$ ) (Table 6.15). There were two major problems: first, the lymphocytes labelled poorly compared to cell lines, leading to a low labelling index and a high supernatant contamination. This phenomenon has been observed by other groups [Coligan, Kruisbeek, Margulies *et al*, 1994]. Second, cell recovery was poor even with an initial population of  $10^7$  cells. A second series of experiments was carried out with modification of the labelling protocol such that the lymphocytes were incubated with a higher concentration of  $^{51}\text{Cr}$  (37 MBq/ml). Cells were exposed to 200kBq  $^{51}\text{Cr}$  in 5 $\mu\text{l}$ , and 1MBq  $^{51}\text{Cr}$  in 25 $\mu\text{l}$  for 1hr. Larger volume washes (4ml) and a different centrifuge (10min at 200g) were used such that the cell pellet formed at the base of the tube rather than slightly to one side, and greater care was taken in aspirating the supernatant. Improvement in cell recovery was highly variable, and did not achieve significance on non-paired *t* test. A parallel set of cells subject to the same washing routine but without exposure to  $^{51}\text{Cr}$  demonstrated similar recovery, and was not dissimilar to that identified during the labelling of cell lines (Table 5.2), suggesting that cell trauma and loss of low buoyant density cells occurring the washing steps was the main problem, rather than toxicity due to the high concentrations of  $^{51}\text{Cr}$  in use. Labelling indices were significantly improved by incubation with 1MBq  $^{51}\text{Cr}$  in 25 $\mu\text{l}$  ( $p < 0.01$ ) though problems with high supernatant contamination persisted. It was considered that perhaps the high ambient  $^{51}\text{Cr}$  concentrations were leading to an intracellular  $^{51}\text{Cr}$  concentration higher than the binding capacity of the cytoplasmic proteins, and that  $^{51}\text{Cr}$  was therefore leaching back out into the supernatant. To test this hypothesis a series of experiments was carried out in which, following incubation with

**Table 6.15 Cr<sup>51</sup> Labelling of Peripheral Blood Lymphocytes.**

<sup>51</sup> Cr	200kBq/100μl	200kBq/5μl	1MBq/25μl	1MBq/25μl <sup>†</sup>
global total	619,311cpm	598,285cpm	2,732,715cpm	1,199,345cpm
cell recovery*	19.9 ± 2.8%	47.2 ± 25.5%	48.0 ± 18.3%	46.3 ± 23.1%
<sup>51</sup> Cr uptake	4.8 ± 1.9%	23.7 ± 9.1%	21.7 ± 5.1%	35.0 ± 6.9%
labelling index	11.8 ± 1.9	35.7 ± 20.4	151.3 ± 50.6	97.0 ± 53.1
Supernatant contamination	20.4 ± 9.0%	31.3 ± 16.8%	23.2 ± 13.8%	2.7 ± 1.1%

*Legend.* Data is expressed as the mean ± standard deviation of 3 experiments. <sup>†</sup>In the absence of <sup>51</sup>Cr the cell recovery was 44.5 ± 30.4%. <sup>‡</sup>Modified washing procedure.

1MBq  $^{51}\text{Cr}$  in 25 $\mu\text{l}$ , the supernatant was removed, and the cells incubated in 4ml of IMDM + 10% FCS for 1hr to achieve a more stable equilibrium, before being subject to the routine washing steps. As proposed, this lead to a slight decrease in labelling index (which was nevertheless superior to that of exposure to 200kBq ( $p<0.05$ )), but a marked reduction in the supernatant contamination (column 5 Table 6.15).

#### 6.7.2 $^{51}\text{Cr}$ -labelling enriched haematopoietic progenitors.

The compound problems associated with high grade HPC enrichment, and in  $^{51}\text{Cr}$ -labelling mononuclear cells did not auger well for the feasibility of applying this approach to HPC. It was decided to optimise the labelling procedure using a standard incubation with 200kBq of  $^{51}\text{Cr}$ . The small numbers of cells available following CD34 $^{+}$  enrichment (range  $5\times 10^4$  to  $5\times 10^5$ ) (Section 6.6.6) made the washing steps particularly problematic because the cell pellet was difficult to visualise. Initial experiments demonstrated that the predominant problems were those of poor cell recovery and viability and difficulty resolving cell labelling above the supernatant contamination. Further experiments with larger volume washes in IMDM + 10% FCS improved the level of supernatant contamination (Table 6.16) allowing definition of the cell labelling index, but did little to improve the poor recovery of viable cells. It seemed clear that a larger initial cell population and an abbreviated enrichment protocol were the main requirements for an effective HPC  $^{51}\text{Cr}$ -labelling protocol.



**Table 6.16 <sup>51</sup>Cr Labelling of Enriched Haematopoietic Progenitors.**

Initial population:  $436 \pm 304 \times 10^3$

Labelled population:  $50 \pm 87 \times 10^3$

Recovery:  $16.4 \pm 23.4\%$

Viability:  $62.5 \pm 22.2\%$

Labelling index:  $22.2 \pm 22.3 \text{cpm}/10^3 \text{ cells}$

Supernatant contamination:  $16.4 \pm 13.2\%$

---

**6.8 Discussion.**

The objective of the work in this Chapter was two fold: first to establish whether the adhesion assay system could be extended to study cell adhesion to stromal tissue cultures, and the extent to which alteration in the nature of the substrate or specific blocking agents could be used to explore the more complex adhesive interactions occurring in this system. Second, it was aimed to establish the feasibility of applying the assay system to bone marrow or peripheral blood derived HPC and to consider the extent to which the purification and Cr<sup>51</sup> labelling of HPC may intrude on the identity and functional behaviour of the study population.

**6.8.1 Long-term bone marrow culture as a model of *in vivo* events.**

A fundamental question is the extent to which LTBMCM can be said to resemble the structure and function of the bone marrow *in vivo*. This aspect has been explored to some extent Section 6.2.1 and only a partial answer can be offered. On the positive side HPC clearly do adhere to marrow endothelium and stroma culture *in vitro*, and stromal layers are capable of supporting sustained proliferation of primitive progenitors over many weeks [Dexter, Wright, Krizsa *et al*, 1977]. This supports the contention that LTBMCM at least supply the adhesive and regulatory elements required for haematopoiesis. On the negative side, the individual elements in LTBMCM are poorly characterised, functional activity is dependent on culture conditions, and the gross structure of the culture cannot be said to resemble normal

bone marrow structure *in vivo*. Caution must therefore be exercised in induction from observations based on LTBMCM to possible mechanisms involved in HPC-stromal interaction *in vivo*.

A second question of substantial import is the extent of redundancy in HPC-stromal adhesion. The presence of a great many potential adhesion pathways and the ineffectual blockade of adhesion to stromal layers using specific agents (as compared to divalent cation chelation) implies that a combined approach to blockade may be required in trying to tease-out the contribution of individual CAMs.

#### 6.8.2 Is the $^{51}\text{Cr}$ adhesion assay applicable to haematopoietic progenitors?

Two key limitations currently prevent the application of the  $^{51}\text{Cr}$ -labelling adhesion assay to HPC. First, the specificity of labelling is entirely dependent on a highly enriched study population. There is conflict between the purity of the  $\text{CD34}^+$  population achieved and the extent to which cell loss and functional alteration degrades confidence that the study population continues to resemble the HPC population *in vivo*. The combination of cell loss and destruction during multiple sequential enrichment procedures and the prolonged duration of *ex vivo* manipulation in the absence of cytokine support lead to loss and functional alteration of HPC which may or may not be selective to certain subsets. For example, physical separation methods may lead to selective loss of large high density HPC, whilst both immunomagnetic / adsorptive and immunocytometric separations are dependent on the intensity of CD34 staining and preferentially retain  $\text{CD34}^{\text{hi}}$  cells. The recovered population may be activated by binding of the mAb and or the bead complex, or cells may have entered apoptosis despite appearing viable on the basis of trypan blue dye exclusion [Koury, 1992]. More subtle influences on target cell proliferation, differentiation or adhesive function remain largely unexplored. Clearly these areas require further detailed scrutiny. It is not easy to achieve sufficient numbers of highly enriched  $\text{CD34}^+$  cells using this protocol. The use of a larger amount of initial material such as an apheresis unit along with a

single step enrichment with a large-volume column would provide a satisfactory number of cells with improved viability albeit at lower final purity.

The second key limitation was the poor sensitivity of the detection system. Peripheral blood mononuclear cells could be labelled to 50-150 cpm/ $10^3$  cells by using high concentrations of  $^{51}\text{Cr}$  and modification of the washing procedure. The central problems with HPC-labelling were related to cell loss rather than difficulty in  $^{51}\text{Cr}$ -labelling *per se*. There are no published protocols successfully  $^{51}\text{Cr}$  labelling enriched HPC. David Leavesley (personal communication) has achieved labelling indices for FACS-sorted  $\text{CD34}^+$  cells in the order of 1,000cpm/ $10^3$  cells by using a custom source of  $^{51}\text{Cr}$  with a specific activity of 740MBq/mg (ml). There is no reason, therefore, that the assay system should not be applicable to HPC populations, provided sufficiently large numbers of enriched cells can be provided with satisfactory viability and functional characteristics.

## **CHAPTER 7. GENERAL DISCUSSION.**

### **7.1 Definition and Analysis of the Haematopoietic Progenitor Population.**

*p 291*

### **7.2 Phenotypic Studies on Haematopoietic Progenitors.**

*p 293*

### **7.3 Functional Studies of Haematopoietic Cell Line Adhesion.**

*p 298*

### **7.4 Functional Assay of Haematopoietic Progenitor Adhesion.**

*p 304*

### **7.5 The Limitations of Current Adhesion Assays.**

*p 306*

*.1 The nature of the carrier and of the test substrate. p 306.*

*.2 Differentiation of adherent and non-adherent populations. p 307.*

*.3 The nature of the assay read-out. p 308.*

*.4 The potential development of dynamic adhesion assays. p 309.*

*.5 Multistep adhesion cascades. p 310.*

*.6 The effects of cell adhesion on proliferation, differentiation and survival. p 312.*

### **7.6 Potential Clinical Applications.**

*p 312*

*.1 Development of less toxic mobilisation regimens. p 312.*

*.2 Ex vivo manipulation of haematopoietic progenitors. p 313.*

### **7.7 Concluding Remarks.**

*p 314*

## 7.1 Definition and Analysis of the Haematopoietic Progenitor Population.

The accuracy and precision with which the HPC population can be defined was considered to be a fundamental issue. The description of haematopoietic stem and progenitor cells is contingent on their potentiality, *i.e.* on their capacity for self-renewal, multilineage proliferation and differentiation, and long-term bone marrow repopulation (Chapter 1.2). In man, semisolid clonogenic assays have proved an invaluable tool for HPC identification and quantitation, but there are some important limitations. First, these assays are retrospective in a formal sense *i.e.* the initiating cell (or cells) are lost as part of the assay. Second, the assay is itself intimately dependent on context. These are fundamental limitations to the informative power of semisolid clonogenic assays when asking questions about cell phenotype and adhesive behaviour. For example it is very difficult to identify and phenotypically characterise the cell or cells which will form clones in a clonogenic assay, and the efficiency with which they will do so may be partially dependent on the presence and nature of substrate adhesion (Chapters 1.2.3 and 4.1)

The CD34 sialomucin demonstrates highly restricted cellular and tissue expression, and is proving valuable as a surrogate marker of the HPC population. The majority of *in vitro* colony forming cells are found within this population and purified CD34<sup>+</sup> populations have proved capable of long-term reconstitution of haematopoiesis and immunity in primates and man *in vivo* [Berenson, Bensinger, Hill *et al*, 1991]. Unfortunately, the small size of the study population and the low density of antigen expression convey significant methodological problems. In this study normal human bone marrow samples (BM) demonstrated a CD34%<sup>+</sup> of  $0.67 \pm 0.6\%$  nucleated cells (Chapter 2.7) with a mean epitope density in the order of 50,000 / cell (Chapter 3.5.2). CD34<sup>+</sup> cells proved undetectable in normal peripheral blood samples (PB) (Chapter 3.2.2), but were present in samples of peripheral blood following high dose chemotherapy and recombinant human granulocyte colony-stimulating factor (PB<sup>m</sup>) at  $2.26 \pm 2.20\%$  (n=8) and in cord blood (CB) at  $0.69 \pm 0.52\%$  (n=4) (Chapter 2.7). These figures are comparable with those of other authors [Bender, Unverzagt, Walker *et al*, 1991; Bender, Williams, Myers *et al*, 1992].



Considerable effort was devoted to the development and optimisation of the CD34 immunocytometry methodology and validation against the CFU-GM clonogenic assay (Chapter 2). It became clear that although CD34 immunocytometry has a number of practical advantages over CFU-GM in terms of generality, rapidity and standardisation, the difficulties in achieving an accurate and reproducible assay remain substantial. The high coefficient of variation of replicate assays, especially at low cell numbers (Chapter 2.7), implies that fundamental technical limitations mitigate against the objective of achieving more reproducible results within and between institutions, despite continued advances in methodology and standardisation (Chapter 2.9.2). Nevertheless, CD34 immunocytometry was considered sufficiently satisfactory to enable pursuit of the phenotypic and functional studies described herein.

A further issue is of central importance to the HPC phenotypic studies of Chapter 3 and the enrichment work of Chapter 6.6, namely the purity and comprehensiveness of the CD34<sup>+</sup> population (discussed in depth in Chapter 2.9.3). Fluorescence overlap between the target and background populations during immunocytometry engenders a conflict between exclusion of false positive cells in the study population and exclusion of true positive cells of low antigen density or unusual light scatter characteristics. In the comparative cell adhesion molecule studies described in Chapter 3.2, contamination of the study population with 10-20% false positive cells was accepted. The availability of superior mAb-fluorochrome conjugates and improvements in immunocytometry methodology in subsequent studies (Chapters 3.3 and 3.5) led to a reduction in contamination to 1-2% allowing more confidence in the validity of the data.

A similar problem was presented in seeking to establish the best method of measuring antigen expression within the CD34<sup>+</sup> population. Here the truism that fluorescence positivity is not synonymous with antigen positivity became important. Fluorescence positivity of the test sample is highly dependent on decisions with regard to placement of discriminatory markers on the negative control, and stringent markers proved to be particularly vulnerable to small numbers of high fluorescence



negative control cells.(Chapter 2.9.3). During the phenotypic HPC studies (Chapter 3) judiciously chosen discriminatory markers and mean fluorescence intensity were found to provide complementary information in clarification of subpopulations of fluorescence positive cells and global shifts in the population fluorescence respectively (Chapter 2.9.3). Studies with calibration beads demonstrated that a linear relationship exists between antigen expression and fluorescence intensity provided a direct labelling protocol is used, and this may prove a very useful additional technique for quantitating antigen expression within the CD34<sup>+</sup> population in the future (Chapter 3.5.2). Unfortunately this system did not give satisfactory results with an indirect labelling protocol and could not be applied to the quantitation of CAM expression during the present study.

## **7.2 Phenotypic Studies on Haematopoietic Progenitors.**

The first and second objectives of this study (Chapter 1.6) directed work to characterise the pattern of cell adhesion molecule (CAM) expression by sessile and circulating HPC, and to attempt to establish whether differences in CAM expression were linked with more generalised differences in lineage and activation marker expression (Chapter 3). The data presented in Chapter 3.2, corroborated the observations of a variety of groups (cited in Chapter 1.6) that HPC express at least 9 cell adhesion molecules belonging to several molecular families. These include immunoglobulin gene superfamily members ICAM-1 (CD54), PECAM-1 (CD31) and LFA-3 (CD58), the  $\beta_1$  integrins VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29), the  $\beta_2$  integrin LFA-1 (CD11a/CD18), L-Selectin (CD62L), the HCAM (CD44) family and CD36 (Chapter 3.2.3). In addition to these HPC express the putative CAMs CD34, CD45, HLA-DR (Chapters 3.2 and 3.3),CD43, Sialyl Lewis<sup>x</sup>, Thy-1 and CD4, as well as cytokine receptors, cell membrane-associated proteoglycans and other poorly characterised molecules such as that described by Tavassoli *et al.* [Referenced in Chapter 1.6].

The differential expression of these molecules was studied on HPC from BM, PB<sup>m</sup> and CB. It was not practical to study all the identified potential adhesion

mechanisms and the study focused on those which are best characterised (Chapter 3.2 and Table 3.1). Equally, the study was not intended to be fully comprehensive with regard to CAMs not normally expressed by BM HPC *i.e.* the possibility that upregulation of one or more CAMs occurs during mobilisation was not studied. The results of this study demonstrated clear reduction in LFA-3 (CD58) and VLA-5 (CD49e/CD29) expression by PB<sup>m</sup> HPC, and reduction in ICAM-1 (CD54), LFA-3 (CD58), VLA-5 (CD49e/CD29), LFA-1 (CD11a/CD18) and L-Selectin (CD62L) by CB HPC. The interpretation of the CB data was complicated by the smaller size of the study population and increased background binding of the second step reagent compared to BM and PB<sup>m</sup> samples (Chapter 3.2.3), making conclusions difficult. The comparative data from the BM and PB<sup>m</sup> work was however sufficiently valid to warrant publication (Appendix 5).

Few studies have asked whether sessile and circulating HPC demonstrate differences in CAM expression. Möhle, Haas & Hunstein, [1993] reported LFA-1 (CD11a/CD18) to be significantly reduced in LP HPC but no difference in ICAM-1 (CD54) or HCAM (CD44) expression, whilst Leavesley, Oliver, Swart *et al* [1994] reported reduced expression of LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29), but not PECAM-1 (CD31) in cryopreserved LP HPC. Saeland, Duvert, Caux *et al* [1992], found no difference in the expression of adhesion molecules between BM and CB CD34<sup>+</sup> cells using a semiquantitative reporting system. The discordance between the results of these studies and those reported herein probably relate to methodological differences and are discussed in some depth in Chapter 3.6.1.

The biological significance of the results of the CAM study remained unclear for two reasons. First, it was uncertain whether differences in CAM expression were a reflection of potential differences in the overall heterogeneity of the CD34<sup>+</sup> population between study populations as a result of source differences or the effects of *in vivo* exposure to chemotherapy or cytokines. Second, as previously recognised, there were serious reservations about using phenotypic differences in CAM expression to infer differences in functional binding behaviour.

It is clear from many immunological and clonogenic studies that the HPC

population defined by CD34<sup>+</sup> is heterogeneous in terms of the stage of differentiation and lineage-committment. In Chapter 3.3 a study was carried out to clarify whether differences in lineage and activation marker expression could be detected between sessile and circulating HPC. The antigens studied were CD33 as a marker of early myeloid differentiation, the transferrin receptor (CD71) - as a marker of erythroid differentiation, CD41a (GpIIb) as a marker of megakaryocytic differentiation, CD7 and CD19 as markers of early T and B lymphoid differentiation respectively. HLA-DR and CD38 were studied as markers of cell "activation" (Chapter 3.3). CD33 and CD19 were found to be expressed by subpopulations of CD34<sup>+</sup> cells within BM from at least some individuals, but not by circulating populations. CD71 was expressed by 40% of BM HPC and somewhat fewer circulating cells. CD38 and HLA-DR were expressed by the majority of HPC, but at higher antigen density in BM compared to circulating populations. There was a particularly marked difference in the expression of CD38 between CB and other populations. In summary, therefore, differences in the state of activation and lineage committment clearly do exist between HPC derived from different sources, suggesting the presence of more mixed CD34<sup>+</sup> population within the BM with more activated cells and erythroid and B-lymphoid precursors (Chapter 3.3.2).

Considerable variation exists between reports from different groups on the levels of antigen positivity or expression. For example the results presented here suggest a very low level of CD33 expression by HPC from all sources, comparable with those of Fritsch, Buchinger, Printz *et al* [1993a], who found CD34<sup>+</sup> cells to be predominantly CD33<sup>-</sup> or CD33<sup>lo</sup>. Others have described significant percentages of CD34<sup>+</sup> CD33<sup>+</sup> cells, albeit at low antigen density and with wide variance between samples [Bender, Unverzagt, Walker *et al*, 1991; Bender, Williams, Myers *et al*, 1992; Saeland, Duvert & Caux 1992; Mohle, Haas & Hunstein 1993] The problem appears to be poor standardisation between research groups with regard to the nature of the study populations, methodology and analytical criteria, and also to expected stochastic variation between studies. In addition, there are clear reservations about interpreting the expression of a single antigen as indicative of a specific

haematopoietic lineage (discussed in depth in Chapter 3.6.2).

It was decided to attempt to determine whether differences in CAM expression occur between identifiable HPC subsets. If so, it was reasoned, differences in CAM expression between sessile and circulating HPC may be explicable on the basis of differences in lineage commitment and activation rather than on the sessile or circulatory origin of the cells. Few studies have attempted to discriminate whether differences exist in CAM expression within CD34<sup>+</sup> subsets. Gunji, Nakamura, Hagiwara *et al.* [1992] have demonstrated differences in LFA-1 expression between primitive and mature CD34<sup>+</sup> cells, whilst Watt, Williamson, Genevier *et al.* [1993] have shown restricted expression of PECAM-1 (CD31) by myeloid and B lymphoid HPC subsets. Several groups have demonstrated lineage and stage specific variation in functional HPC adhesion to cellular and non-cellular substrates using clonogenic assays, supporting the likelihood that differences in HPC subset expression of CAM do exist (cited in Chapter 1.6). Two approaches were taken to address this issue: immunocytometry studies on human haematopoietic cell lines (Chapter 3.4) and the development of three colour immunocytometry of HPC (Chapter 3.5).

Single colour immunocytometry was used to study the expression of the CAMs, lineage and activation markers detailed above, in a group of human haematopoietic cell lines chosen to represent a variety of stages and lineages of differentiation: the acute myeloblastic leukaemia line KG1a, the chronic myeloid leukaemia line K562, the acute promyelocytic leukaemia line HL60, the acute T lymphoblastic leukaemia line CEM and the B lymphoblastic leukaemia line NALM-6. This simple study was quite informative. It was clear that the expression of lineage and activation markers did not correspond particularly well with the derivation of the lines (Chapter 3.4). For example none of the myeloid lines expressed CD33, whereas both KG1a and CEM expressed CD7 and K562 and NALM-6 expressed CD19. CD71 was expressed by all lines except HL60, underlining its widespread expression on proliferating cells, whilst CD38 was only expressed on the lymphoid cell lines. Multiparameter immunocytometric analysis of peripheral blood leucocyte populations has illustrated that antigen expression is rarely lineage-restricted and is



better described as lineage-associated [Terstappen, Hollander, Meiners *et al*, 1990]. This study served to emphasise that lineage marker associations deduced from scrutiny of normal haematopoietic precursors and mature cells cannot be assumed to be valid when applied to neoplastic haematopoietic cells, haematopoietic cell lines and HPC [Greaves, Chan, Furley *et al*. 1986].

Whilst some CAMs were expressed on all lines (CD54, CD49d and CD49e), others were differentially expressed (CD58, CD31, CD11a, CD51, CD44 and CD36) or not at all (CD62L). Clustering of CAMs and lineage / activation markers could be demonstrated, but did not fall easily into a preconceived pattern. Of relevance, LFA-3 was expressed by all lines except CEM, and VLA-5 by all lines, making it less likely that expression of these antigens is affected by differences in lineage heterogeneity.

The only way to resolve the issue of differential expression of CAM within HPC lineage subsets is by three colour immunocytometry of CD34<sup>+</sup> subpopulations (Chapter 3.5). This represents a formidable technical challenge because of the composite problems described above. Several preliminary studies were carried out to optimise the methodology. A comparative study of available third colour reagents showed that both Quantum Red and PerCP provided reasonable discrimination between positive and negative test populations, though the latter provided a simpler labelling protocol (Chapter 3.5.1). The use of calibration beads allowed the amount of antigen present on the cell surface to be quantified, provided that a direct labelling technique was used. Indirect technique led to poor discrimination between individual bead populations and degraded linearity in the relationship between antigen density and fluorescence (Chapter 3.5.2). However it did prove reasonably straightforward to acquire samples on the basis of CD34<sup>+</sup>/FSC (Chapter 3.5.3), and this proved the key to providing sufficient cells to enable three colour analysis and the enrichment work discussed later (Chapter 6.6 and Section 7.4). Subanalysis of CD33<sup>+</sup>, CD7<sup>+</sup>, CD19<sup>+</sup> or CD41a<sup>+</sup> HPC subsets either in terms of light scatter characteristics (Chapter 3.5.4) or CAM expression was not a practical proposition because of the very small numbers of cells involved. However, it was possible to

discriminate CD71, HLA-DR and CD38 positive and negative subpopulations, which were also the most relevant because these markers demonstrated the greatest differences between sessile and circulating CD34<sup>+</sup> populations (*vide supra*). Expression of CD58 and CD49e was examined within CD34<sup>+</sup> CD71<sup>+/</sup>, CD34<sup>+</sup> HLA-DR<sup>+/</sup> and CD34<sup>+</sup> CD38<sup>+/</sup> subpopulations. Allowing for the technical limitations of the study, LFA-3 (CD58) expression did not vary significantly between subsets, whilst VLA-5 (CD49e) expression was greater in the CD34<sup>+</sup> CD71<sup>+</sup> and the CD34<sup>+</sup> CD38<sup>+</sup> populations. Since CD71 and CD38 were more highly expressed by BM than PB<sup>m</sup> CD34<sup>+</sup> cells, this data supports the proposition that the reduced VLA-5 expression in the circulating CD34<sup>+</sup> population is a reflection of other changes in HPC activation or differential mobilisation of some subsets.

### **7.3 Functional Studies of Haematopoietic Cell Line Adhesion.**

Cell adhesion molecule expression is a necessary but insufficient criterion from which to conclude participation in HPC adhesion, because expression does not necessarily confer functional activity. Many CAM occur as multiple isoforms (*e.g.* CD54 and CD44) or must undergo membrane redistribution or conformational change to achieve maximum avidity (*e.g.* CD49d and CD49e). The development of an assay to study functional HPC adhesion was therefore essential to evaluate the potential role of the CAMs described above in HPC homing and engraftment, and to consider whether reduction in CD49e expression in particular could be responsible for HPC mobilisation (Chapter 1.6). Adhesion assays based on direct visualisation and clonogenic assay suffer from similar limitations in accuracy namely the statistical variation in the distribution of small numbers of cells (which conform with a Poisson distribution) and high observer errors (Chapter 4.1). Clonogenic assays are additionally dependent on the culture environment, making it sometimes difficult to separate conditions affecting adhesion from those affecting cloning efficiency (Chapters 1.2.3 and 4.1). The <sup>51</sup>Cr adhesion assay offered the potential advantages of larger numbers of cells, the correlation of phenotypic and functional data, and applicability to the study of adhesion of HPC and haematopoietic cell lines



to a variety of cellular and non-cellular substrates (Chapter 4). This type of assay was used by Hardy and Minguell (1993) to study murine haematopoietic cell line adhesion to fibroblast cell lines. The  $^{51}\text{Cr}$  adhesion assay was developed using human haematopoietic cell lines in the first instance, because these provided the advantages of the availability of large numbers of cells, of a relatively consistent and homogeneous population within which lineage differentiation and adhesion molecule expression had already been partially defined (Chapter 3.4 and *vide supra*). KG1a and purified plasma fibronectin (pFn) were used to develop and optimise the assay system. Briefly,  $10^7$  KG1a were labelled by incubation with 200kBq of  $^{51}\text{Cr}$  for 1hr at ambient temperature. The  $^{51}\text{Cr}$  was removed and the cells washed twice, leading to a labelling index (LI) of around 40cpm/ $10^3$  cells (Chapter 4.2). It was calculated that at this level of labelling a minimum of  $10^5$  cells could be used per assay well to resolve down to 1% of the study population. In practice approximately  $2.5 \times 10^5$  cells were used per well. Optimal conditions for coating the plastic carrier (*i.e.* a 24 well tissue culture plate) with fibronectin were established: a 1hr incubation at ambient temperature with 100 $\mu\text{l}$  of 50 $\mu\text{g}/\text{ml}$  was found to be satisfactory (Chapter 4.3). During the adhesive phase of the experiment, labelled KG1a were incubated in the substrate-coated wells for 2 hours at ambient temperature. During the distractive phase, the supernatant was removed by pipette, the well was washed twice with medium and the supernatant and washes were pooled (Chapter 4.4). The adherent cells were lysed using a detergent in distilled water. Both the adherent and the non-adherent fractions were then counted in a gamma counter, and the total count and adherence percentage calculated. Denatured bovine serum albumin (dBSA) coated wells were used as a negative control because of the high background binding of cells to uncoated tissue culture plastic. Assays and controls were established in triplicate within a particular 24 well plate and the mean percentage adherence of the three wells was taken as the assay result. The extent of cellular adherence was found to be particularly dependent on the stringency of the washing procedure following the adhesion phase of the experiment and this was standardised as far as possible. Monitoring of a series of experiments over 6-12

months confirmed an acceptable degree of assay reproducibility (Chapter 4.4.6). In addition, overall labelling and adhesion parameters were similar to those of Hardy & Minguell [1993] and of John Sweetenham and Lisa Masek in Southampton who have used the assay in collaborative work (*vide infra* and also Chapter 6.1 and Appendix 4).

The  $^{51}\text{Cr}$  adhesion assay was used in a series of studies to examine the adhesion of the haematopoietic cell lines detailed above to human extracellular matrix (ECM) components (Chapter 5). The commercially purified components studied were collagens types I, III and IV, fibronectin, vitronectin, thrombospondin and vitronectin, and heparan sulphate, chondroitin sulphate and hyaluronic acid. These proteins, glycoproteins and proteoglycans are known to be expressed within human bone marrow stroma [Gordon, 1988a; Clark, Gallagher & Dexter, 1992]. Differences in the functional binding of haematopoietic cell lines to ECM components were demonstrated (Chapter 5.2) and some correlations with cell line CAM expression were apparent (Chapter 3.4). For example, all five cell lines express VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29) and all demonstrated significant binding to pFn. KG1a express CD36 and bound markedly to thrombospondin. Important areas of discordance were also demonstrated. For example CD31<sup>+</sup> CD45<sup>+</sup> KG1a did not adhere to heparan sulphate, whereas CD31<sup>-</sup> CD45<sup>-</sup> NALM-6 did. Similarly KG1a, HL60 and CEM express HCAM (CD44) but adhered neither to collagens nor to hyaluronic acid. K562 express VNR (CD51) though they did not adhere to vitronectin.

The information required to predict functional adhesion behaviour on the basis of CAM expression is necessarily incomplete. A full characterisation of CAM expression by the cell lines studied was not carried out and additional molecules may therefore augment cell adhesion behaviour. In addition, the functional activity of some of the CAMs known to be expressed (Chapter 3.4) could not be fully assessed in the assay system because the appropriate ligands are predominantly cell surface bound and were not readily available in purified form. Finally, it is clear that CAM expression is not sufficient in itself to convey adhesion to an appropriate

ligand. Some CAMs may be expressed in a non-activated form, or may mediate low avidity adhesion obscured by the relatively high shear stress imparted during the distractive phase of the assay.

Several approaches were taken to extend these observations:

First, a phorbol ester and a calcium ionophore were used to "activate" KG1a in order to establish whether some CAMs were expressed in a non-activated state (Section 5.3). Although the ionophore had little effect, the phorbol ester markedly increased adhesion to glycoproteins and proteoglycans, and also background binding to dBSA. This could be due to an increase in the expression or avidity of multiple CAMs, or downregulation of a global negative regulator of adhesion in response to phorbol ester. Background binding to dBSA was also found to be variable during general experimentation (Chapter 4.4.6) - an observation confirmed by other workers in the field (David Leavesley, personal communication). How one should interpret these findings is not clear, but clearly KG1a-pFn adhesion is not simply dependent on CAM expression, but also on their state of "activation" and on negative adhesion molecules *i.e.* molecules which function to control non-specific cell adhesion.

Second, a variety of approaches were used to attempt to blockade KG1a adhesion to pFn as a model system in which to establish whether the correlation between CAM expression and functional binding to the appropriate ligand was sufficient to conclude a causal link between the two (Chapter 5.4). Several different agents were used to explore this interaction including divalent cation chelation with HBSS and EDTA, a synthetic tetrapeptide known to mimic the recognition motif on fibronectin for VLA-5 (CD49e/CD29) (RGDS), mAbs known to blockade VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29) activity in other experimental systems, and a series of enzymes known to degrade proteoglycans. Partial blockade of KG1a-pFn adhesion was achieved by divalent cation chelation, a 5mM solution of RGDS and by chondroitinase ABC, but not by the other agents examined. Clearly, therefore KG1a adhesion to pFn is partially dependent on VLA-5 but also on chondroitin sulphate, and potentially also on other mechanisms (combination

experiments were not carried out).

Third, the assay system was extended to examine the adhesion of haematopoietic cell lines to bone marrow stromal and endothelial cultures *in vitro* in order to mimic some of the complexity of HPC-stromal interaction occurring *in vivo* and also to examine in more depth the proposition that reduction or blockade of VLA-5 (CD49e/CD29) activity might alone be sufficient to explain HPC mobilisation. To extend the assay system in this way further developmental work was undertaken (Chapter 6). Long-term bone marrow stromal cultures were prepared using the techniques first described for mice by Dexter, Allen & Lajtha [1977], and later adapted to human marrow by Gartner and Kaplan [1980]. Aliquots of sterile bone marrow were cultured for 3 weeks in a medium containing foetal calf and horse serum and hydrocortisone. The confluent stromal layer that forms over this period is complex, containing monocytes, fibroblasts, adipocytes and endothelial cells, and is capable of sustaining primitive haematopoietic progenitors which proliferate to form large discrete colonies or "cobblestone areas" (Chapter 6.2). Preparatory experiments demonstrated the need to grow the cultures on collagen to prevent disruption of the integrity of the stromal layer during the distractive phase of the adhesion assay (Chapter 6.3). KG1a adhesion to long-term bone marrow stromal cultures, bone marrow fibroblast cultures (grown without horse serum or hydrocortisone) and a murine bone marrow fibroblast cell line (M210-B4) was examined and found to be similar to each cellular substrate and significantly greater than that to pFn (Chapter 6.4). Blockade of KG1a adhesion to M210-B4 was therefore examined using divalent cation chelation, synthetic peptides, enzymes and a broadened panel of mAbs to ICAM-1 (CD54), PECAM-1 (CD31), LFA-1 (CD11a) and HCAM (CD44) as well as those to VLA-4 (CD49d) and VLA-5 (CD49e). Only divalent cation chelation engendered any significant degree of blockade in this system, suggesting that although integrins as a group were essential to KG1a-stromal adhesion, individual CAMs in the system were redundant. Miyake *et al* [Miyake, Medina, Hayashi *et al.* 1990; Miyake, Weissman, Greenberger *et al.* 1991] have demonstrated inhibition of lymphohaematopoiesis in



murine long-term bone marrow stromal cultures by blockade of the VLA-4 / VCAM-1 and the CD44 / hyaluronate pathways. However it is not clear whether inhibition of HPC adhesion to the stroma, or impediment of the survival or proliferation of HPC or their progeny was the mechanism responsible for these observations.

The adhesion of human haematopoietic cell lines to human umbilical vein endothelial and bone marrow endothelial cultures was studied in collaborative work with John Sweetenham and Lisa Masek of the University of Southampton (Appendix 4). All five cell lines adhered significantly to endothelial cultures *in vitro*. Adherence of KG1a, HL60 and CEM was significantly greater to umbilical vein endothelium (HUVEC) activated with tumour necrosis factor- $\alpha$  than to non-activated, whilst K562 and NALM-6 demonstrated no difference. There was no difference in the adherence of any of the cell lines between activated and non-activated endothelial LTBM, but adherence of KG1a, HL60 and CEM to endothelial LTBM was superior to that to non-activated HUVECS, and on a par with that to activated HUVECS. It appears, therefore, that some cell lines (KG1a, HL60, CEM) but not others (K562, NALM-6) are capable of responding to activation of endothelium by tumour necrosis factor  $\alpha$ , and that bone marrow endothelial cultures are constitutively activated. These differences may be helpful in clarifying changes in ligand expression of functional importance during endothelial activation. The study also demonstrates that the  $^{51}\text{Cr}$  adhesion assay is applicable not just to examine the effects of HPC activation and blockade on stromal and endothelial adhesion, but also to examine the effects of manipulation of the cellular substrate .

In summary of the functional adhesion work on haematopoietic cell lines, it can be said that the  $^{51}\text{Cr}$  adhesion assay has been shown to provide a satisfactory method for addressing questions about the relationship between HPC cell adhesion molecule expression and functional adhesion behaviour, and that manipulation of the study population and/or of the substrate by activation or blockade can help to dissect the

composite mechanisms responsible for adhesion to the stromal microenvironment.

It has also been demonstrated that HPC adhesion to the bone marrow stroma *in vivo* is unlikely to be wholly based on any individual cell adhesion molecule - ligand pathway such as VLA-5 (CD49e/CD29) interaction with fibronectin. The observed reduction in VLA-5 expression on circulating compared to sessile HPC is not sufficient in itself to explain HPC mobilisation.

#### **7.4 Functional Assay of Haematopoietic Progenitor Adhesion.**

In the final part of the thesis it was decided to examine whether the  $^{51}\text{Cr}$  assay could be applied directly to the study of human HPC. This brought together the developmental work on the immunocytometric definition and characterisation of HPC (Chapters 2 and 3) with that on the  $\text{Cr}^{51}$  adhesion assay (Chapters 4-6).

As a first step it was clear that highly enriched populations of HPC would have to be prepared, and a three-step enrichment protocol was designed entailing the use of leucapheresis or density-gradient prepared cells, a preparatory enrichment using the *miniMACS* column and a final enrichment using a fluorescence activated cell sorter (Chapter 6.6). Cells were labelled with an anti-CD34-RPE mAb and a RAM-supraparamagnetic microbead second step reagent using established techniques (Chapters 2.4 and 6.6). Up to  $10^8$  cells were added to the top of a *miniMACS* column in a powerful magnetic field and allowed to flow through under the influence of gravity. The eluate was retrieved by removing the column from the magnetic field and flushing with fresh medium. The *MACS* eluate was then FACSorted.  $\text{CD34}^+$  cells were acquired through an open gate designed to exclude debris and cell aggregates only, and were discriminated on the basis of a 0.1% marker established on a negative control sample (Chapter 3.5 & 3.6, and 6.6.). They were enumerated during collection by the FACSorter, the percentage positivity in the final product was assessed by passing an aliquot through the FACScan, and viability of the final product was assessed by trypan blue dye exclusion. The composite results demonstrated a final purity of 82% with a viability of >90% and an 65 fold enrichment factor. The final yield of purified  $\text{CD34}^+$  cells from  $69 \times 10^6$  LP was



0.25x10<sup>6</sup> cells, representing a recovery of 19%. It was not feasible to determine whether selective loss of HPC subsets had occurred, although both the *MACS* and the *FACSort* favour the selective retention of CD34<sup>hi</sup> HPC. The enrichment protocol took approximately 6hrs to complete, and although the cells looked normal on microscopic examination and were mainly viable on trypan blue exclusion, there was no way of knowing whether they may have undergone more subtle changes in function.

As a second step the labelling of peripheral blood mononuclear cells with <sup>51</sup>Cr was examined prior to application to enriched HPC (Chapter 6.7.1). Peripheral blood mononuclear cells labelled poorly with standard concentrations of <sup>51</sup>Cr, but satisfactory labelling could be achieved by using high concentrations of <sup>51</sup>Cr and a modified washing procedure. Additional problems were encountered in attempting to <sup>51</sup>Cr-label enriched HPC (Chapter 6.7.2). The small numbers of cells available following CD34<sup>+</sup> enrichment made the washing steps particularly difficult because the cell pellet was difficult to visualise. The recovery from this procedure was 16%, leading to a labelled population of 5.0x10<sup>4</sup>, with a viability of 62% (trypan blue dye exclusion), a labelling index of 22cpm/10<sup>3</sup> cells and a supernatant contamination of 16%. There are no published protocols successfully <sup>51</sup>Cr labelling enriched HPC though David Leavesley (personal communication) has achieved labelling indices for FACS-sorted CD34<sup>+</sup> cells in the order of 1,000cpm/10<sup>3</sup> cells by using a custom source of <sup>51</sup>Cr with a specific activity of 740MBq/mg (ml).

In summary, therefore, it is feasible to prepare <sup>51</sup>Cr-labelled HPC for adhesion experiments but the use of a larger amount of initial material such as an apheresis unit is required along with an abbreviated enrichment protocol. A single step enrichment with a large-volume column should provide a satisfactory cell yield and viability, albeit providing a product with lower final purity. Combining this approach with a high intensity <sup>51</sup>Cr labelling protocol should provide sufficient cells for functional adhesion experiments. More work is required to establish the effects of cell enrichment and labelling on CD34<sup>+</sup> subsets and function.

## **7.5 The Limitations of Current Adhesion Assays and Their Possible Resolution.**

The key problems and limitations of current HPC adhesion assays have been discussed within the relevant sections of the text, and are summarised here as a focal point for discussion of the direction of future research.

### *7.5.1 The nature of the carrier and of the test substrate.*

The nature of the carrier is critical to satisfactory binding and presentation of the substrate. Tissue culture plastics are thought to bind proteins and cells predominantly through ionic and hydrophobic interactions, increasing the background against which specific binding must be discriminated. Some companies such as Costar now provide a range of 96 well plates with well characterised surfaces. It is possible that some ligands will display preferential adsorption to particular types of plastic, or that the orientation or binding avidity will be further affected by the nature of the carrier. It is probably desirable to define the optimal concentration and binding conditions for each ligand and carrier (Chapter 4.3.2).

The source and purity of the substrate is also of importance to the validity of the assay. Contamination of the test substrate with other proteins or carbohydrates may affect the result of the assay. Many ECM components occur in multiple isoforms which vary depending on the source of the material. In the assays carried out in the course of these studies, commercial human materials of defined purity were used wherever possible, although some proteoglycans were of bovine origin (Appendix 1). Moreover, commercial preparations do show variation between companies, and those derived from miscellaneous human tissues may differ structurally from those present in human bone marrow stroma (see for example the discussion of Fn isoforms in Chapter 5.4 and 5.5). The optimal policy would be to purify study materials direct from the relevant tissue in house, such that contamination and structural variation could be more closely defined.

Denatured BSA is a satisfactory control despite a significant amount of batch-to-batch variation in cellular binding (Chapter 4.4 and 4.5). This problem has been also reported by other workers in the field (David Leavesley, personal communication),

and there is no easy resolution - the only clear alternative being to use a purified protein to which one is confident the test cells will not bind (for example: collagen type IV in the case of haematopoietic cell lines).

#### *7.5.2 Differentiation of adherent and non-adherent populations.*

The critical issue in the measurement of cell-substrate adhesion is control over the shear forces applied during the distractive phase of the adhesion assay. Removal of non-adherent cells by pipette is difficult to control and may be of such magnitude as to largely obscure low avidity binding interactions such as that between selectins and proteoglycans, whilst preserving high avidity interactions such as those involving integrin binding to ECM glycoproteins. One approach worthy of exploration in future studies would be to reduce shear forces by converting the assay to a 96 well plate system, in which case one could distract non-adherent cells by inverting the plate for an hour. Gravity provides a uniform distraction force and the fluid does not fall out of the wells due to surface tension. The top 50% of the supernatant can then be removed with a multiwell pipette.

A related, though somewhat conceptual issue, is that there is often no clear way of discriminating whether the results of a particular adhesion assay reflect the overall adhesive affinity and capacity of a homogeneous cell population for a given substrate, or the behaviour of two or more subpopulations with qualitatively different adhesive properties. In many cases these complementary interpretations of the data are equally valid ways of viewing the data (see the discussion of the problems of formulating the results of adhesion assays in Chapter 4.5, and similar problems with immunocytometry data in Chapter 2.9). A system in which serial assays could be carried out at varying concentration may allow deduction of affinity and adhesive capacity of the substrate for the study population as a whole through a Scatchard analysis (Chapter 4.4.4).

### *7.5.3 The nature of the assay read-out.*

Direct enumeration of the adherent population by normal light or phase contrast microscopy can be applied to cell lines or highly enriched HPC populations on non-cellular substrates. Fluorescence microscopy has wider applicability to non- or partially-enriched HPC populations, on cellular and non-cellular substrates.

Although these approaches are the most direct, they suffer from several limitations with regard to stochastic distribution and observer variation as discussed in Chapter 4.1. Semisolid clonogenic assays have been used by several groups to study HPC adhesion mechanisms. This kind of assay is useful in that it yields information on adhesive behaviour of functionally defined HPC subpopulations. The limitations are firstly, that the clonogenic assay employed is not necessarily representative of the adhesive behaviour of other HPC subpopulations, or of HPC as a whole. It may be necessary to combine a panel of clonogenic assays, or to focus on a functionally-defined HPC subpopulation. Secondly, that the phenotypic identity of the cell giving rise to a particular colony is unknown, making it difficult to correlate the behaviour of the adhesive cells with their cell adhesion molecule expression. Thirdly, that alteration of HPC cloning efficiency may occur due to the presence of accessory cells or as a direct result of substrate adhesion. Fourthly, that the assays are difficult to standardise between laboratories, are labour intensive and time consuming.

Although the  $^{51}\text{Cr}$ -adhesion assay has proved satisfactory in the analysis of cell line adhesion, where large numbers of cells of a fairly homogeneous phenotype are available, it is clearly less easily applicable to the study of HPC for the reasons outlined above (Section 7.4). To reiterate, the problems are two-fold: the production of sufficient numbers of highly purified HPC and the achievement of a satisfactory labelling index, with the addendum that the study population may undergo physiologically significant functional alteration during the preparatory procedures. Although the initial problems can be overcome given sufficiently large amounts of harvest, resource and enthusiasm, it is by no means clear that the addendum can be satisfied. There is some advantage in the use of highly purified HPC populations for adhesion studies, namely the elimination of accessory cells which may inhibit HPC



adhesion through competition for available binding sites, steric hindrance or secretion of cytokines which alter the functional behaviour of the study population. More detailed analysis of the product of intensive cell purification in terms of pattern of differentiation, proliferation and apoptosis is clearly required. One alternative might be to convey specificity within the labelling / read-out system (rather than separating the two functions as in CD34-purification and  $^{51}\text{Cr}$ -labelling). For example, if the study population were labelled with an anti-CD34 mAb and a second step reagent before carrying out the adhesion phase of the assay, a read-out could then be achieved using an enzymatic (colorimetric or chemiluminescence) or fluorimetric method following separation of the adherent and non-adherent populations. A potential advantage of this type of approach is that it may be easier to amplify the detection system in order to improve the sensitivity into the  $10^2$  to  $10^4$  range. This would permit analysis of non- or partially-purified, as well as highly purified CD34 $^+$  populations, and would permit conversion of the assay to a 96 well plate system, with the consequent advantages in assay turnover, informative content and standardised supernatant removal.

#### 7.5.4 *The potential development of dynamic adhesion assays.*

Up to this point, consideration has been restricted to adhesion assays which are essentially static in nature. There are a number of important limitations in the relevance of such assays, particularly *a propos* the interaction of HPC with endothelium under vascular flow conditions. In principle, vascular flow limits the time available for an HPC to interact with an endothelial cells before moving-on, and also provides a shear force which serves to distract low affinity adhesions. The time taken for the cells to sediment to the test surface, and the absence of detailed control over shear force are absolute limitations in most static assay systems.

A number of strategies have been developed to try to overcome these problems. Leavesley, Oliver, Swart *et al.* [1994], have used a flow cytometry based assay to study interaction of CD34 $^+$  cells with CHO cells stably transfected with human ICAM-1 and VCAM-1. The test cells are incubated together in suspension on a

roller, fixed following various periods, and analysed for the loss of single positive cells due to formation of CD34<sup>+</sup>-CHO doublets and higher aggregates. In a similar approach, Aizawa *et al.* have studied formation of rosettes between stromal and haematopoietic cells [Aizawa, Hojoh, Suda *et al.* 1991] The advantage of this kind of approach is that the cell-cell interaction can be arrested at any stage, allowing analysis of early events, and that the low shear forces invoked allow assessment of low avidity binding. A disadvantage is that it is difficult to accurately quantitate the shear force applied.

Nordon, Milthorpe, Schindhelm *et al* [1994] have developed a system to examine the strength of cell-ligand affinity based on cell detachment at a critical uniform shear stress generated by controlling flow rate through a 100µm parallel-plate system. The narrow plate separation allowed complete cell sedimentation within 30s, and permitted study of the early stages of bond formation. Flow residence time, median critical shear stress, temperature and ligand density, were found to influence cell attachment probability. Attachment strength was found to be a heterogeneous property due to cellular heterogeneity and to the stochastic fluctuation inherent in an adhesive bond dependent on small numbers [1,000-6,000] of marker / ligand interactions. Lackie [1991] has developed a similar approach in which cells in suspension pass over endothelium in a chamber with well defined linear flow characteristics. The shear force is a combination of the velocity of flow (controlled by a pump) and the viscosity of the medium. Cell adhesion over a discrete area of the surface is recorded by video. These kind of distraction and flow assays have not yet been applied to HPC studies.

#### *7.5.5 Multistep adhesion cascades.*

A further issue arises from these studies. Given that the majority of the CAMs described are expressed by diverse cell types and the widespread distribution of ECM molecules such as fibronectin and thrombospondin, how is tissue specificity of adhesion conveyed? The concept of multistep adhesion cascades has been advanced by several authors [Butcher, 1991 & 1993; Tanaka & Shaw, 1992;



Tanaka, Albelda, Horgan *et al.* 1992; Springer, 1994]. These authors suggest that leucocyte-homing requires a highly specific (but low avidity) recognition system which mediates initial interaction with vascular endothelial molecules (addressins) imparting positional information. In lymphocyte homing a lectin has been described which recognises phosphomannose residues displayed by high endothelial venules [Gallatin, St John, Sieglemen *et al.* 1986]. The HPC-marrow endothelium homing receptor is unknown, though Tavassoli's group have demonstrated expression of a lectin that recognises mannose / galactose residues [Aizawa & Tavassoli, 1987; 1988], and PECAM-1 (CD31), HCAM (CD44), CD45 and L-Selectin (CD62L) all display carbohydrate specificities. The initial recognition event is postulated to trigger amplification of the adhesive response through activation of high-avidity, low specificity adhesion systems, such as those mediated by integrins. Leavesley *et al.* have demonstrated that VLA-4 (CD49d/CD29) mediated adhesion of CD34<sup>+</sup> cells to VCAM-1 (CD106), but not LFA-1 (CD11a/CD18) mediated adhesion to ICAM-1 (CD54), is enhanced by antibodies to co-expressed PECAM-1 (CD31) [Leavesley, Oliver, Swart *et al.* 1994]. Majdic *et al.* have demonstrated that mAbs to O-sialoglycoprotease-sensitive epitopes of CD34 (Section 1.4) can activate the LFA-1:ICAM-1 adhesion system through protein tyrosine kinases, and that this requires cellular energy and cytoskeletal reorganisation [Majdic, Stöckl, Pickl *et al.* 1994]. Low avidity interactions are difficult to study in current adhesion assays because of poor control over the shear forces generated in distracting the non-adherent population as discussed above. Adhesion of cell lines to extracellular matrix components is partially integrin mediated (Chapters 5.2 and 5.4) and is not dependent on protein synthesis, metabolic energy or cytoskeletal reorganisation (Chapter 5.4.3), suggesting that these molecules are expressed in a functionally active form. The effects of PMA on the adhesive behaviour of cell lines (Chapter 5.3) suggests either that these molecules can be further activated, or that negative regulators of adhesion exist which have hitherto been largely ignored. Clearly more subtle adhesion assays are required if these sequential events are to be elucidated.

#### *7.5.6 The effects of cell adhesion on proliferation, differentiation and survival.*

Recently, it has become clear that adhesive interactions between HPC and extracellular matrix components may in part be responsible for modulation of cellular proliferation, differentiation or apoptosis. Catherine Verfaillie has demonstrated that direct contact between HPC and stroma is not essential to HPC survival, but in the absence of such contact excessive proliferation occurs [Verfaillie, 1992]. More recently the group has provided evidence that adhesion of HPC to fibronectin inhibits cellular proliferation [Hurley & Verfaillie, 1993] and has further suggested that the stromal adhesion defect seen in chronic myeloid leukaemia blasts may contribute to their uncontrolled proliferation [Verfaillie, McCarthy & McGlave, 1992]. Long-term maintenance of primitive human HPC in culture requires unidentified stromal-derived factors in addition to known stimulatory and inhibitory cytokines [Verfaillie, 1994] probably including ECM components like fibronectin, thrombospondin [Long & Dixit, 1990; Long, Briddell, Walter *et al.* 1992] and proteoglycans [Sponcer, Gallagher, Krizsa *et al.* 1983; Roberts, Gallagher, Sponcer *et al.* 1988; Gordon, Riley & Clarke, 1988; Bruno, Guscar, Luikart *et al.* 1993; Gupta, McCarthy & Verfaillie, 1993]. Once again we have barely scratched the surface in studying the effects of adhesion on HPC kinetics.

### **7.6 Potential Clinical Applications.**

Two potential applications of this study were thought to be worthy of further consideration.

#### *7.6.1 Development of less toxic HPC mobilisation regimens.*

It was considered possible that the intravenous use of peptide fragments or blocking mAbs may permit development of less toxic regimens for mobilisation of HPC from the bone marrow. Papayannopoulou & Nakamoto [1993] have recently provided experimental evidence that an anti-VLA-4 (CD49d) mAb infused into primates produced up to a 200 fold rise in HPC in the peripheral blood. The use of

RGD-containing fragments was an attractive proposition because these can be easily synthesised, and are likely to be non- immunogenic *in vivo*. There are two limitations to the approach. First, the data from Chapters 5.4 and 6.5 mitigates against the possibility of blocking HPC-stromal adhesion *in vivo* using a single agents. Second, at concentrations high enough to blockade HPC-Fn adhesion, synthetic peptides also interfere with other RGD-dependent adhesive interactions such as that between platelet GpIIb/IIIa (CD41a/CD61) and fibrinogen. This possibility was explored using a platelet aggregation assay. Briefly, platelet rich plasma was prepared by centrifugation of whole blood at 10,000rpm for 10 min, and platelet poor plasma by centrifugation at 30,000rpm for 10 min. These two solutions were used to calibrate measurement of minimum and maximum light transmission by a spectrophotometer. Aliquots of platelet rich plasma were incubated for 30 min in the presence or absence of 5mM RGDS, and then stimulated with 5 $\mu$ M ADP, 2.8 $\mu$ g/ml collagen, 0.5u/ml thrombin, 1.5mg/ml ristocetin or 500 $\mu$ g/ml arachadonic acid. RGDS virtually abolished platelet aggregation in response to ADP, and significantly inhibited that to thrombin and ristocetin [Gartner & Bennett, 1985]. This data suggests that RGDS would not be satisfactory as an *in vivo* mobilising agent because the peptide would precipitate a haemorrhagic diathesis. Many toxic snake venoms contain this same binding motif [Hutton & Warrell, 1993]. Slightly longer peptides containing amino acids adjacent to the RGD sequence may convey greater specificity to VLA-5 and may be of clinical utility if the specificity is sufficiently discrete. [Pierschbacher & Ruoslahti, 1987; Hautanen, Gailit, Mann *et al.* 1989].

#### 7.6.2 *Ex vivo manipulation of haematopoietic progenitors.*

Stromal cultures have been used in the clinical setting to support HPC during *ex vivo* manipulation, to augment HPC gene transfection and to purge neoplastic cells from contaminated bone marrow or peripheral blood harvests [Gordon, Dowding, Riley *et al.* 1987; Verfaillie, McCarthy & McGlave, 1992]. There are a number of practical disadvantages to this technique. First, autologous stroma can only be

derived from bone marrow and so co-culture of peripheral blood or cord blood HPC requires a preformed autologous or allogeneic stroma. Second, the stroma is fragile and requires a rigid carrier. In effect, this restricts stromal culture to flasks in an open system, increasing the environmental exposure of the harvest. Third, the stromal cultures are poorly characterised and the system is therefore difficult to standardise or control. One way of mitigating these problems would be to construct blood-bag plastics with an ECM coating. Flexible blood-bags provide an enclosed, gas-permeable system with a non-adherent surface in which clinical harvests can be handled, reducing environmental exposure but allowing *ex vivo* manipulation including culture and cryopreservation. Judicious construction of a matrix may allow selective retention of HPC, controlled *ex vivo* culture with removal of mature cells, neoplastic cell purging, or HPC gene transfection. In addition, the work of Verfaillie *et al* [Hurley & Verfaillie, 1993], suggests that ECM components which until recently have been considered to be relatively inert, play an active role in control of cell physiological functions. Preliminary work in this area has been carried out and is currently the subject of a patent application.

## **7.7 Concluding Remarks.**

The objective of the work presented within this thesis was the development of assay techniques which would enable the study of human haematopoietic progenitor adhesive interactions with the bone marrow microenvironment, and to investigate the mechanisms involved in HPC migration, mobilisation and homing. Immunocytometry has provided valuable information on the range of cell adhesion molecules expressed by HPC, and has suggested that differences exist between sessile and circulating progenitors both in terms of activation and lineage commitment and CAM expression. Advanced immunocytometry holds the promise of being able to dissect CAM expression within very low frequency subpopulations of HPC and of being able to purify those subpopulations for functional studies. Functional adhesion assays are essential to a fuller understanding of the relevance of phenotypic studies. The  $^{51}\text{Cr}$  assay presented here represents a step in the direction

of drawing causal links between CAM expression and functional cellular binding behaviour. Clearly much technical work is required in the development of this and other approaches to the study of HPC adhesion, and the dissection of individual adhesion pathways by activation and blockade. The difficulty in identifying discrete HPC subpopulations within what appears to be a continuum, the plethora of adhesion mechanisms, the categorical overlap between adhesion and communication mechanisms, and the dependence of HPC identity on environmental conditions, suggests a depth of physiological complexity which a homeostatic metaphor ill-equips us to understand [Bernard, 1865]. The challenge presented is to put the individual pieces together, not simply as a study in bone marrow transplantation, but as an amenable model system with which to develop a fuller understanding of human morphogenesis [Thompson, 1961].



## BIBLIOGRAPHY.

Aizawa S, Tavassoli M (1987). In vitro homing of hemopoietic stem cells is mediated by a recognition system with galactosyl and mannosyl specificities. *Proceedings of the National Academy of Sciences USA* 84: 4485-4489.

Aizawa S, Tavassoli M (1987). Interaction of murine granulocyte-macrophage progenitors and supporting stroma involves a recognition system with galactosyl and mannosyl specificities. *Journal of Clinical Investigation* 80: 1698-1705.

Aizawa S, Tavassoli M (1988). Detection of membrane lectins on the surface of hemopoietic progenitor cells and their changing pattern during differentiation. *Experimental Hematology* 16: 325-329.

Aizawa S, Tavassoli M (1988). Molecular basis of the recognition of intravenously transplanted hemopoietic cells by bone marrow. *Proceedings of the National Academy of Sciences USA* 85: 3180-3183.

Aizawa S, Tavassoli M (1988). Marrow uptake of galactosyl-containing neoglycoproteins: implication in stem cell homing. *Experimental Hematology* 16: 811-813.

Aizawa S, Hojo H, Tsuda A, Sai M, Toyama K (1991). Rosette formation between stromal and hemopoietic cells. A simple assay for the supportive activity of stromal cells. *Leukaemia* 5: 273-276.

Aizawa S, Yaguchi M, Nakano M, Inokuchi S, Handa H, Toyama K (1991). Establishment of a variety of human bone marrow stromal cell lines by recombinant SV-40 adenovirus vector. *Journal of Cellular Physiology* 148: 245-251.

Akira S, Hirano T, Taga T, Kishimoto T (1990). Biology of multifunctional cytokines: IL-6 and related molecules (IL-1 and TNF). *FASEB Journal* 4: 2860 - 2867.

Akiyama SK, Yamada SS, Chen WT, Yamada KM (1989). Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organisation. *Journal of Cell Biology* 109: 863-875.

Akiyama SK, Larjava H, Yamada KM (1990). Differences in the biosynthesis and localization of the fibronectin receptor in normal and transformed cultured human cells. *Cancer Research* 50: 1601-1607.

Albelda SM, Buck CA. (1990). Integrins and other cell adhesion molecules. *FASEB Journal* 4, 2868-2880.



Albelda SM, Muller WA, Buck CA, Newman PJ (1991). Molecular and cellular properties of PECAM-1 (endoCAM/CD31): A novel vascular cell-cell adhesion molecule. *Journal of Cell Biology* 114: 1059-1068.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1989). Cell adhesion, cell junctions, and the extracellular matrix. *In: Molecular Biology of the Cell*. 2nd Ed. Garland Publishing Inc. New York: 792-834.

Allen TD, Dexter TM (1983). Long term bone marrow cultures: an ultrastructural review. *Scanning Electron Microscopy* 4: 1851-1866.

Allen TD, Dexter TM (1984). The essential cells of the hemopoietic microenvironment. *Experimental Hematology* 12: 517-521.

Allen TD, Dexter TM, Simmons PJ (1990). Marrow biopsy and stem cells. *In: Dexter TM, Garland JM, Testa NG eds. Hematology, Vol IX. Colony Stimulating Factors: Molecular and Cellular Biology*. Marcel Dekker, New York. 1-38.

Altman DG (1994). *Practical Statistics for Medical Research*. Chapman & Hall, London.

Anderson C, Looney R (1986). Human leucocyte IgG Fc receptor. *Immunology Today* 7: 264-266.

Anderson CJ, Looney RJ (1987). Immunoglobulin G Fc receptors of human leukocytes. *Methods in Enzymology* 150: 524-536.

Andrews RG, Singer JW, Bernstein ID (1989). Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of CD33 and CD34 antigens and light scattering properties. *Journal of Experimental Medicine* 169: 1721-1731.

Andrews RG, Singer JW, Bernstein ID (1990). Human hematopoietic precursors in long term culture: single CD34<sup>+</sup> cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony forming cells when cultured with marrow stromal cells. *Journal of Experimental Medicine* 172: 355-358.

Arkin S, Naprstek B, Guarini L, Ferrone S, Lipton JM (1991). Expression of intracellular molecule-1 (CD54) on hematopoietic progenitors. *Blood* 77: 948-953.

Arnaout MA (1990). Structure and function of leukocyte adhesion molecules CD11/CD18. *Blood* 75: 1037-1050.

Asch AS, Barnwell J, Silverstein RL, Nachman RL (1987). Isolation of the thrombospondin membrane receptor. *Journal of Clinical Investigation* 79: 1054-1061.

Asch AS, Tepler J, Silbiger S, Nachman RL (1991). Cellular attachment to thrombospondin: cooperative interactions between receptor systems. *Journal of Biological Chemistry* 266: 1740-1745.

Atkinson K (1990). Reconstruction of the haemopoietic and immune systems after marrow transplantation. *Bone Marrow Transplantation* 5: 209-226.

Barrett AJ, Gordon MY (1993). *Bone Marrow Disorders. The Biological Basis of Treatment*. Blackwell Scientific Publications, Oxford.

Barrett AJ, Longhurst P, Sneath P, Watson JG (1978). Mobilization of CFU-C by exercise and ACTH induced stress in man. *Experimental Hematology* 6: 590-594.

Baum CM, Weissman IL, Tsukamoto AS, Buckle AM, Peault B (1992). Isolation of a candidate human hematopoietic stem-cell population. *Proceedings of the National Academy of Sciences, USA* 89: 2804-2808.

Baumhueter S, Singer MS, Henzel W, Hemmerich S, Renz M, Rosen SD, Lasky LA (1993). Binding of L-Selectin to the vascular sialomucin CD34. *Science* 262: 436-438.

Baumhueter S, Dybdal N, Kyle C, Lasky LA (1994). Global vascular expression of murine CD34, a sialomucin-like ligand for L-Selectin. *Blood* 84: 2554-2565.

Bell GI, Dembo M, Bongrand P (1984). Cell adhesion: competition between non-specific repulsion and specific binding. *Biophysical Journal* 45, 1051-1064.

Bender JG, Unverzagt KL, Walker DE, Lee W, Van Epps DE, Smith DH, Stewart CC, To LB (1991). Identification and comparison of CD34 positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. *Blood* 77: 2591-2596.

Bender JG, Williams SF, Myers S, Nottleman D, Lee WJ, Unverzagt KL, Walker D, To LB, Van Epps DE (1992). Characterization of chemotherapy mobilized peripheral blood progenitor cells for use in autologous stem cell transplantation. *Bone Marrow Transplantation* 10: 281-285.

Bender JG, To LB, Williams S, Schwartzberg LS (1992). Defining a therapeutic dose of peripheral blood stem cells. *Journal of Hematotherapy* 1: 329-341.

Bender JG, Lum L, Unverzagt KL, Lee W, Van Epps D, George S, Coon J, Ghalie R, McLeod B, Kaizer H, Williams SF (1994). Correlation of colony-forming cells, long-term culture initiating cells and CD34<sup>+</sup> cells in apheresis products from patients mobilized for peripheral blood progenitors with different regimens. *Bone Marrow Transplantation* 13: 479-485.

Bender JG, Unverzagt K, Walker D (1994). Guidelines for determination of CD34<sup>+</sup> cells by flow cytometry: application to the harvesting and transplantation of peripheral blood stem cells. *In: Wunder E, Sovalat H, Hénon PR, Serke S eds. Hematopoietic Stem Cells: The Mulhouse Manual. AlphaMed Press, Dayton, Ohio: 31-44.*

Bender JG, Unverzagt K, Walker DE, Lee W, Smith S, Willimas S, Van Epps DE (1994). Phenotypic analysis and characterization of CD34<sup>+</sup> cells from normal human bone marrow, cord blood, peripheral blood, and mobilized peripheral blood cells from patients undergoing autologous stem cell transplantation. *Clinical Immunology & Immunopathology* 70: 10-18.

Bentley SA (1982). Collagen synthesis by bone marrow stromal cells: a quantitative study. *British Journal of Haematology* 50: 491-497.

Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID (1988). Antigen CD34<sup>+</sup> marrow cells engraft lethally irradiated baboons. *Journal of Clinical Investigation* 81: 951-955.

Berenson RJ, Bensinger WI, Hill RS, Andrews RG, Garcia-Lopez J, Kalamasz DF, Still BJ, Spitzer G, Buckner CD, Bernstein ID, Thomas ED (1991). Engraftment after infusion of CD34<sup>+</sup> marrow cells in patients with breast cancer or neuroblastoma. *Blood* 77: 1717-1722.

Bernard C (1865). *Introduction a l'etude de la medecine experimentale*. Bailliere: Paris.

Bertoncello I, Bradley TR, Hodgson GS, Dunlop JM (1991). The resolution, enrichment, and organisation of normal bone marrow high proliferative potential colony-forming subsets on the basis of rhodamine 123 fluorescence. *Experimental Hematology* 19: 174-178.

Bertoncello I, Bradley TR, Watt SM (1991). An improved negative immunomagnetic selection strategy for the purification of primitive hemopoietic cells from normal bone marrow. *Experimental Hematology* 19: 95-100.

Bevilacqua MP, Nelson RM (1993). Selectins. *Journal of Clinical Investigation* 91: 379-387.

Blackett NM (1974). Statistical accuracy to be expected from cell colony assays; with special reference to the spleen colony assay. *Cell and Tissue Kinetics* 7: 407-412.

Blazsek I, Liu XH, Anjo A, Quittet P, Comisso M, Kim-Triana B, Misset JL (1995). The hematon, a morphogenic functional complex in mammalian bone marrow, involving erythroblastic islands and granulocytic cobblestones. *Experimental Hematology* 23: 309-319.

Bradley TR, Metcalf D (1966). The growth of mouse bone marrow cells in vitro. *Australian Journal of Experimental Biology and Medical Science* 44: 287-299.

Bradley TR, Hodgson GS, Rosendaal M (1978). The effect of oxygen tension on hematopoietic and fibroblast cell proliferation in vitro. *Journal of Cell Physiology* 97: 517-522.

Brecher G, Beal SL, Schneiderman M (1986). Renewal and release of hemopoietic stem cells: does clonal succession exist? *Blood Cells* 12: 103-127.

Brenner MK, Rill DR, Moen RC, Krance RA, Mirro J, Anderson WF, Ihle JN (1994). Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. *Lancet* 341: 85-86.

Briddell RA, Broudy VC, Bruno E, Brandt JE, Srouf EF, Hoffman R (1992). Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to the *c-kit* receptor. *Blood* 79: 3159-3167.

Brown J, Greaves MF, Molgaard HV (1991). The gene encoding the stem cell antigen CD34 is conserved in mouse and expressed in haemopoietic progenitor cell lines, brain and embryonic fibroblasts. *International Immunology* 3: 175-184.

Broxmeyer HE, Douglas GW, Hangoc G, Cooper S, Bard J, English D, Army M, Thomas L, Boyse EA (1989). Human umbilical cord blood as a potential source of transplantable hematopoietic stem / progenitor cells. *Proceedings of the National Academy of Sciences USA*, 86: 3828-3832.

Broxmeyer HE (1991). Self-renewal and migration of stem cells during embryonic and fetal hematopoiesis: important but poorly understood events. *Blood Cells* 17: 282-286.

Broxmeyer HE, Kurtzberg J, Gluckman E, Auerbach AD, Douglas G, Cooper S, Falkenburg JHF, Bard J, Boyse EA (1991). Umbilical cord blood hematopoietic stem and repopulating cells in human clinical transplantation. *Blood Cells* 17: 313-329.

Broxmeyer HE, Hangoc G, Cooper S (1992). Clinical and biological aspects of human umbilical cord blood as a source of transplantable hematopoietic stem and progenitor cells. *Bone Marrow Transplantation*: 9 (Suppl 1): 7-10.

- Bruno E, Guscar T, Luikart S, Wilson E, Dixit V, Long M, Hoffman R (1993). Role of heparan sulphate in modulating human hematopoietic stem cell attachment to cytokines and extracellular matrix molecules. *Blood* 82: 21 *abstract*.
- Buck CA, Horowitz AF (1987). Cell surface receptors for extracellular matrix molecules. *Annual Reviews in Cell Biology* 3: 179-205.
- Burroughs J, Gupta P, Blazar BT, Verfaillie CM (1994). Diffusible Factors from the murine cell line M2-10B4 support human in vitro hematopoiesis. *Experimental Hematology* 22: 1095-1101.
- Butcher EC, Ford WC (1986). Following cellular traffic : methods of labelling lymphocytes and other cells to trace their migration in vivo. *In*: Weir DM, Herzenberg LA, Blackwell C, Herzenberg LA *eds*. *Handbook of Experimental Immunology*. Vol 1. Immunochemistry. 4th Ed. Blackwell Scientific Publications, Oxford: 57.1-57.23.
- Butcher EC (1991). Leukocyte-endothelial cell recognition: three or more steps to specificity and diversity. *Cell* 67: 1033-1036.
- Butcher EC (1993). Specificity of leukocyte-endothelial interactions and diapedesis: physiologic and therapeutic implications of active decision process. *Research in Immunology* 144: 695-698.
- Caldwell CW, Taylor HM (1986). A rapid, no-wash technic for immunophenotypic analysis by flow cytometry. *American Journal of Clinical Pathology* 86: 600-607.
- Caldwell CW, Patterson WP, Toalson BD, Yesus YW (1991). Surface and cytoplasmic expression of CD45 antigen isoforms in normal and malignant myeloid cell differentiation. *American Journal of Clinical Pathology* 95: 180-187.
- Campbell AD, Long MW, Wicha MS (1987). Haemonectin, a bone marrow cell adhesion protein specific for cells of granulocytic lineage. *Nature* 329: 744-746.
- Campbell AD, Long MW, Wicha MS (1990). Developmental regulation of granulocytic cell binding to hemonectin. *Blood* 76: 1758-1764.
- Chabannon C, Torok-Storb B (1992). Stem cell-stromal cell interactions. *Current Topics in Microbiology and Immunology* 177: 123-136.
- Charbord PM, Lerat H, Newton I, Tamayo E, Gown AM, Singer JW, Hervé P (1990). The cytoskeleton of stromal cells from human bone marrow cultures resembles that of cultured smooth muscle cells. *Experimental Hematology* 18: 276-282.



Chaudhary PM, Roninson IB (1991). Expression and activity of P- glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell* 66: 85-94.

Chervenick PA, Boggs DR (1971). In vitro growth of granulocytic and mononuclear cell colonies from blood of normal individuals. *Blood* 37: 131-135.

Chichester CO, Fernandez M, Minguell JJ (1993). Extracellular matrix gene expression by human bone marrow stroma and by marrow fibroblasts. *Cell Adhesion and Communication* 1: 93-99.

Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *Journal of Immunology* 133: 157-165.

Civin CI, Banquerigo ML, Strauss LC, Loken MR (1987). Antigenic analysis of hematopoiesis VI. Flow cytometric characterization of My-10 positive progenitor cells in normal human bone marrow. *Experimental Hematology* 15: 10-17.

Civin CI, Loken MR (1987). Cell surface antigens on human bone marrow cells: dissection of hematopoietic development using monoclonal antibodies and multiparameter flow cytometry. *International Journal of Cell Cloning* 5: 267-288, 1987.

Civin CI, Trischmann TM, Fackler MJ, Bernstein ID, Bühring HJ, Campos L, Greaves MF, Kamoun M, Katz DR, Lansdorp PM, Look AT, Seed B, Sutherland DR, Tindle RW, Uchanska-Ziegler B (1989). Report on the CD34 cluster workshop. *In: Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr eds. Leucocyte Typing IV; White Cell Differentiation Antigens.* Oxford University Press, Oxford: 818-825.

Civin C, Strauss LC, Fackler MJ, Trischmann TM, Wiley JM, Loken MR (1990). Positive stem cell selection - basic science. *In: Bone Marrow Purging and Processing.* Alan R Liss Inc, New York: 387- 401.

Civin C (1992). Identification and positive selection of human progenitor / stem cells: for bone marrow transplantation. *Progress in Clinical and Biological Research* 377: 461-472.

Clark BR, Gallagher JT, Dexter TM (1992). Cell adhesion in the stromal regulation of haemopoiesis. *In: Lord BI, Dexter TM eds. Growth Factors in Haemopoiesis; Clinical Haematology* 5. Ballière Tindall, London: 619-652.

Clayberger C, Krensky AM, McIntyre BW, Koller TD, Parham P, Brodsky F, Linn DJ, Evans EL (1987). Identification and characterisation of two novel lymphocyte-function associated antigens L24 and L25. *Journal of Immunology* 138: 1510-1514.



Cole-Sinclair M, Foroni L, Wright F, Mehta A, Prentice HG, Hoffbrand AV (1993). Minimal residual disease in acute lymphoblastic leukaemia - PCR analysis of immunoglobulin gene rearrangements. *Leukaemia and Lymphoma* 11 (Sp 2): 49-58.

Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (1994). *Current Protocols in Immunology* 1: 7.28.1-22.

Collins SJ, Gallo RC, Gallacher RE (1977). Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* 270: 347-349.

Conget P, Minguell JJ (1994). Modifications in the synthesis of membrane-associated chondroitin sulfate proteoglycans in hemopoietic progenitor cells are associated with alterations in their adhesive properties. *Journal of Cellular Physiology* 159: 142-150.

Coombe DR, Watt SM, Parish CR (1994). Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitors cells to stromal cell elements via recognition of stromal heparan sulphate. *Blood* 84: 739-752.

Cosman D, Lyman SD, Idzerda RL, Beckmann MP, Park LS, Goodwin RG, March CJ (1990). A new cytokine receptor superfamily. *Trends in Biochemical Sciences* 15: 265-270.

Coulombel L, Eaves AC, Eaves CJ (1983). Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. *Blood* 62: 291-297.

Coulombel L, Vuillet-Gaugler MH, Leroy C, Tchernia G (1988). Lineage and stage specific adhesion of human hematopoietic progenitor cells to extracellular matrices from fibroblasts. *Blood* 71: 329-334.

Coulombel L, Vuillet-Gaugler MH, Leroy C, Roseblatt M, Breton Gorius J (1991). Adhesive properties of human erythroblastic precursor cells. *Blood Cells* 17: 65-81.

Craig JIO, Turner ML, Parker AC (1992). Peripheral blood stem cell transplantation. *Blood Reviews* 6: 59-67.

Craig JIO, Smith SM, Parker AC, Anthony RS (1992). The response to peripheral blood stem cells to standard chemotherapy for lymphoma. *Leukaemia and Lymphoma* 6: 363-368.

Craig JIO, Anthony RS, Stewart A, Thomson EB, Gillon J, Parker AC (1993). Peripheral blood stem cell mobilisation using high dose cyclophosphamide and G-CSF in pretreated patients with lymphoma. *British Journal of Haematology* 85: 210-212.

Craig JI, Langlands K, Parker AC, Anthony RS (1994). Molecular detection of tumour contamination in peripheral blood stem cell harvests. *Experimental Hematology* 22: 898-902.

Craig W, Thomas TE, Lansdorp PM (1992). Use of lectins for characterisation and purification of human bone marrow cells that express CD34. *Journal of Hematotherapy* 1: 55-64.

Craig W, Kay R, Cutler RL, Lansdorp PM (1993). Expression of Thy-1 on human hematopoietic progenitor cells. *Journal of Experimental Medicine* 177: 1331-1342.

Craig W, Poppema S, Little MT, Dragowska W, Lansdorp PM (1994). CD45 isoform expression on human haemopoietic cells of different stages of development. *British Journal of Haematology* 88: 24-30.

Dacie JV, Lewis SM (1975). *Practical Haematology*. Fifth Edition. Churchill Livingstone, Edinburgh.

Dainiak N (1991). Surface membrane-associated regulation of cell assembly, differentiation, and growth. *Blood* 78: 264-276.

Danpure HJ, Osman S (1990). Methods of radiolabelling blood cells. *In: Textbook of Radiopharmacy. Theory and Practice*. Sampson CB *ed*. Gordon and Breach Publishers, New York: 209-228.

De Lisser HM, Newman PJ, Albelda SM (1994). Molecular and functional aspects of PECAM-1 / CD31. *Immunology Today* 15: 490-495.

Delia D, Lampugnani MG, Resnati M, Dejana E, Aiello A, Fontanella E, Soligo D, Pierotti MA, Greaves MF (1993). CD34 expression is regulated reciprocally with adhesion molecules in vascular endothelial cells in vitro. *Blood* 81: 1001-1008.

Dexter TM, Allen TD, Lajtha LG (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. *Journal of Cellular Physiology* 91: 335-344.

Dexter TM, Wright EG, Krizsa F, Lajtha LG (1977). Regulation of haemopoietic stem cell proliferation in long term bone marrow cultures. *Biomedicine* 27: 344-349.

Dexter TM, Spooncer E, Simmons PJ, Allen TD (1984). Long-term marrow culture: an overview of techniques and experience. *In: Wright DG, Greenberger JS eds. Long-term bone marrow culture*. Alan R Liss Inc, New York: 57-96.

Dexter TM, Coutinho LH, Spooncer E, Heyworth CM, Daniel CP, Schiro R, Chang J, Allen TD (1990). Stromal cells in haemopoiesis. 1990 Molecular control of haemopoiesis. Wiley, Chichester (Ciba Foundation Symposium 148): 76-95.

Diamond MS, Springer TA (1994). The dynamic regulation of integrin adhesiveness. *Current Biology* 4: 506-517.

Diem K, Lentner C *eds* (1970). *Documenta Geigy: Scientific Tables*. Seventh Edition. Geigy, Basel. 558.

Dougherty GJ, Lansdorp PM, Cooper DL, Humphries RK (1991). Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. *Journal of Experimental Medicine* 174: 1-5.

Dransfield I, Cabanas C, Craig A, Hogg N (1992). Divalent cation regulation of the function of the leukocyte integrin LFA-1. *Journal of Cell Biology* 116: 219-226.

Eaves CJ, Sutherland HJ, Udomsakdi C, Lansdorp PM, Szilvassy SJ, Fraser CC, Humphries RK, Barnett MJ, Phillips GL, Eaves AC (1992). The human hematopoietic stem cell in vitro and in vivo. *Blood Cells* 18: 301-307.

Eaves CJ, Cashman JD, Kay RJ, Dougherty GJ, Otsuka T, Gaboury LA, Hogge DE, Lansdorp PM, Eaves AC, Humphries RK (1991). Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. II. Analysis of positive and negative regulators produced by stromal cells within the adherent layer. *Blood* 78: 110-117.

Edelman GM (1987). CAMs and Igs: cell adhesion and the evolutionary origins of immunity. *Immunological Reviews* 100, 11- 45.

Edelman GM (1988). *Topobiology: an introduction to molecular embryology*. Basic Books Inc. New York.

Egeland T, Steen R, Tjonnfjord GE, Ostrem R, Boretti E, Gaudernack G (1993). Monoclonal antibodies against different epitopes of the CD34 molecule. *Experimental Hematology* 21: 1135 *abstract*.

Egeland T, Tjonnfjord G, Steen R, Gaudernack G, Thorsby E (1993). Positive selection of bone marrow-derived CD34 positive cells for possible transplantation. *Transplantation Proceedings* 25: 1261-1263.

Egeland T, Gaudernack G (1994). Immunomagnetic isolation of CD34<sup>+</sup> cells: methodology and monoclonal antibodies. *In*: Wunder E, Sovalat H, Hénon PR, Serke S *eds*. *Hematopoietic Stem Cells: The Mulhouse Manual*. AlphaMed Press, Ohio, USA. 141-148.

Ehrlich P (1879). Ueber die specifischen Granulationen des Blutes. *Archiv fur Physiologie, Leipzig*. 571-579.

Eliason JF, Testa NG, Dexter TM (1979). Erythropoietin-stimulated erythropoiesis in long-term bone marrow culture. *Nature* 281: 382-384.

Emerson SG, Artin JH (1989). Bone marrow progenitor cells induce a regulatory autologous proliferative T lymphocyte response. *Journal of Immunology* 142: 766-772.

Fausser AA, Messner HA (1979). Identification of megakaryocytes, macrophages and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. *Blood* 53: 1023-1027.

Ferreira O-C, Valinsky JE, Sheridan K, Wayne EA, Bianco C, Garcia-Pardo A (1991). Phorbol-ester induced differentiation of U937 cells enhances attachment to fibronectin and distinctly modulates the  $\alpha 5\beta 1$  and  $\alpha 4\beta 1$  fibronectin receptors. *Experimental Cell Research* 193: 20-26.

Fibach E, Gerassi E, Sachs L (1976). Induction of colony formation in vitro by human lymphocytes. *Nature* 259: 127-129.

Flanagan CJ, Mitoma TF (1958). Clumping (false agglutination) of blood from the umbilical cord. *American Journal of Clinical Pathology* 29: 337-339.

Flemming S (1990). Cellular Functions of Adhesion Molecules. *Journal of Pathology* 161, 189-190.

Ford CE, Hamerton JL, Barnes DWH, Loutit JF (1956). Cytological identification of radiation chimeras. *Nature* 177: 452-454.

Foxwall C, Watson SR, Dowbenko D, Fennie C, Lasky LA, Kiso M, Hasegawa A, Asa D, Brandley BK (1992). Three members of the Selectin family recognise a common carbohydrate epitope, the sialyl Lewis<sup>x</sup> oligosaccharide. *Journal of Cell Biology* 117: 895-902.

Fraser JK, Leahy MF, Berridge MV (1986). Expression of antigens of the platelet glycoprotein IIb-IIIa complex on human hematopoietic stem cells. *Blood* 68: 762-769.

Freshney RI (1987). *Culture of Animal Cells. A Manual of Basic Technique*. 2nd Ed. Wiley-Liss Inc. New York.

Freshney RI, Pragnell IB, Freshney MG (1994). *Culture of Hematopoietic Cells*. Wiley-Liss, New York.

Fritsch G, Emminger W, Buchinger P, Printz D, Gadner H (1991). CD34-positive cell proportions in peripheral blood correlate with colony-forming capacity. *Experimental Hematology* 19: 1079-1083.



Fritsch G, Buchinger P, Printz D, Strunk D, Agis H, Sliutz G, Wilfing A, Schulz U, Gadner H (1993a). Contradictory results with CD33 expression on CD34 positive hematopoietic progenitor cells. 3rd International Symposium on Peripheral Blood Stem Cell Autografts: *abstract*.

Fritsch G, Buchinger P, Printz D, Fink FM, Mann G, Peters C, Wagner T, Adler A, Gadner H (1993b). Rapid discrimination of early CD34+ myeloid progenitors using CD45RA analysis. *Blood* 81: 2301-2309.

Fritsch G, Buchinger P, Printz D (1993c). Use of flow cytometric CD34 analysis to quantify hematopoietic progenitor cells. *Leukaemia and Lymphoma* 10: 443-451.

Gale RP (1992). Fetal liver transplants. *Bone Marrow Transplantation* 9 (Suppl 1): 118-120.

Gallatin M, St John TP, Siegelman M, Reichert R, Butcher EC, Weissman IL (1986). Lymphocyte homing receptors. *Cell* 44: 673-680.

Gallin JI, Clark RA, Kimball HR (1973). Granulocyte chemotaxis: an improved in vitro assay employing <sup>51</sup>Cr-labeled granulocytes. *Journal of Immunology* 110: 233-240.

Galmiche MC, Koteliansky VE, Brière J, Hervé P, Charbord P (1993). Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. *Blood* 82: 66-76.

Gamble JR, Vadas MA (1988). A new assay for the measurement of the attachment of neutrophils and other cell types to endothelial cells. *Journal of Immunological Methods* 109: 175-184.

Gartner S, Kaplan HS (1980). Long-term culture of human bone marrow cells. *Proceedings of the National Academy of Sciences, USA* 77: 4756-4759.

Gartner TK, Bennett JS (1985). The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. *The Journal of Biological Chemistry* 260: 11891-11894.

Gay RE, Prince CW, Zuckerman KS, Gay S (1989). The collagenous hemopoietic microenvironment. In: Tavassoli M, *ed*. *Handbook of the Hemopoietic Microenvironment*. Humana Press, New Jersey: 369-398.

Gee AP, Lamb LS (1994). Preparation of samples for flow cytometric analysis: recommendations from the ISHAGE course in San José. In: Wunder E, Sovalat H, Hénon PR, Serke S *eds*. *Hematopoietic Stem Cells: The Mulhouse Manual*. AlphaMed Press, Ohio: 67-78.

- Giancotti FG, Comoglio PM, Tarone G (1986). Fibronectin-plasma membrane interaction in the adhesion of hemopoietic cells. *Journal of Cell Biology* 103: 429-437.
- Gjessing R, Seglen PO (1980). Adsorption, simple binding and complex binding of rat hepatocytes to various in vitro substrates. *Experimental Cell Research* 129: 239-249.
- Glazer AN, Stryer L (1983). Fluorescent tandem phycobilirubin conjugates; emission wavelength shifting by energy transfer. *Biophysical Journal* 43: 383-386.
- Golding JW (1983). Monoclonal antibodies: principles and practices. Production and application of monoclonal antibodies in cell biology, biochemistry and immunology. Academic Press Inc, New York.
- Gordon MY, Riley GP, Watt SM, Greaves MF (1987). Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature* 326: 403-405.
- Gordon MY, Dowding CR, Riley GP, Goldman JM, Greaves MF (1987). Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. *Nature* 328: 342-344.
- Gordon MY, Riley GP, Clarke D (1988). Heparan sulfate is necessary for adhesive interactions between human early hemopoietic progenitor cells and the extracellular matrix of the marrow microenvironment. *Leukaemia* 2: 804-809.
- Gordon MY (1988a). Extracellular matrix of the marrow microenvironment. *British Journal of Haematology* 70: 1-4.
- Gordon MY (1988b). Adhesive properties of haemopoietic cells. *British Journal of Haematology*. 68: 149-151.
- Gordon MY, Greaves MF (1989). Physiological mechanisms of stem cell regulation in bone marrow transplantation and haemopoiesis. *Bone Marrow Transplantation* 4: 335-338.
- Gordon MY, Clarke D, Atkinson J, Greaves MF (1990). Hemopoietic progenitor cell binding to the stromal microenvironment in vitro. *Experimental Hematology* 18: 837-842.
- Gordon MY (1991). Hemopoietic growth factors and receptors: bound and free. *Cancer Cells* 3; 127-133.
- Gordon MY (1993a). Human haemopoietic stem cell assays. *Blood Reviews* 7: 190-197.



- Gordon MY (1993b). Perspective: physiological mechanisms in BMT and haemopoiesis - revisited. *Bone Marrow Transplantation* 11: 193-197.
- Gordon MY (1994). Physiology and function of the haemopoietic microenvironment. *British Journal of Haematology* 86: 241-243.
- Gore SD, Kastan MB, Civin CI (1991). Normal human bone marrow precursors that express terminal deoxynucleotidyl transferase include T cell precursors and possible lymphoid stem cells. *Blood* 77: 1681-1690.
- Graham IL, Brown EJ (1991). Extracellular calcium results in a conformational change in MAC-1 (CD11b/CD18) on neutrophils. Differentiation of adhesion and phagocytic functions of MAC-1. *Journal of Immunology* 146, 685-691.
- Greaves MF, Chan LC, Furley AJW, Watt SM, Molgaard HV (1986). Lineage promiscuity in hematopoietic differentiation and leukaemia. *Blood* 67: 1-11.
- Greaves MF, Brown J, Molgaard HV, Spurr NK, Robertson D, Delia D, Sutherland DR (1992). Molecular features of CD34: a hemopoietic progenitor cell-associated molecule. *Leukaemia* 1992; 6 (Suppl 1): 31-36.
- Greenberger JS (1978). Sensitivity of corticosteroid-dependent insulin-resistant lipogenesis in marrow preadipocytes of obese diabetic (db/db) mice. *Nature* 275: 752-754.
- Greenberger JS (1986). Future directions in bone marrow transplantation: interests converge on the bone marrow microenvironment. *British Journal of Haematology* 62: 603-605.
- Greenberger JS (1991). Toxic effects on the hematopoietic microenvironment. *Experimental Hematology* 19: 1101-1109.
- Greenwald I, Rubin GM (1992). Making a difference: the role of cell-cell interactions in establishing separate identities for equivalent cells. *Cell* 68: 271-281.
- Greenwalt JS, Lipsky RH, Ockenhouse CF, Ikeda H, Tandon NN, Jamieson GA (1992). Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* 80: 1105-1115.
- Gronthos S, Graves SE, Ohta S, Simmons PJ (1994). The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. *Blood* 84: 4164-4173.
- Groopman JE, Molina JM, Scadden DT (1989). Hematopoietic growth factors. *New England Journal of Medicine* 321: 1449-1459.

- Gunji Y, Nakamura M, Hagiwara T, Hayakawa K, Matsushita H, Osawa H, Nagayoshi K, Nakauchi H, Yanagisawa M, Miura Y, Suda T (1992). Expression and function of adhesion molecules on human hematopoietic stem cells: CD34<sup>+</sup> LFA-1<sup>-</sup> cells are more primitive than CD34<sup>+</sup> LFA-1<sup>+</sup> cells. *Blood* 80: 429-436.
- Gupta P, McCarthy JB, Verfaillie CM (1990). Stroma-derived, anionic macromolecule(s) in combination with subliminal amounts of cytokines support the in vitro maintenance and differentiation of human primitive LTC-IC. *Blood* 82: 73 *abstract*.
- Hardy CL, Matsuoka T, Tavassoli M (1991). Distribution of homing protein on hemopoietic stromal and progenitor cells. *Experimental Hematology* 19: 968-972.
- Hardy CL, Minguell JJ (1993). A cytoadhesion assay for the binding of cloned hemopoietic progenitor cells to stroma. *Experimental Hematology* 21: 283-288.
- Harrison DE, Astle CM, Lerner C (1988). Number and continuous proliferative pattern of transplanted primitive immunohematopoietic stem cells. *Proceedings of the National Academy of Sciences USA* 85: 822-826.
- Harrison DE, Jordan CT, Zhong RK, Astle CM (1993). Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Experimental Hematology* 21: 206-219.
- Hautanen A, Gailit J, Mann DM, Ruoslahti E (1989). Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor. *Journal of Biological Chemistry* 264: 1437-1442.
- Hayflick L (1965). The limited in vitro lifetime of human diploid cell strains. *Experimental Cell Research* 37: 614-636.
- Haylock DN, To LB, Dowse TL, Makino S, Trimboli S, Niutta S, Juttner CA, Simmons PJ (1994). Ex vivo expansion of purified stem cells. *In: Wunder E, Sovalat H, Henon Ph, Serke S eds. Hematopoietic Stem Cells. The Mulhouse Manual. AlphaMed Press, Ohio: 291-302.*
- Haynes BF, Telen MJ, Hale LP, Denning SM (1989). CD44 - A molecule involved in leukocyte adherence and T cell activation. *Immunology Today* 10: 423-428.
- Haynes BF, Liao HX, Patton KL (1991). The transmembrane hyaluronate receptor (CD44): multiple functions, multiple forms. *Cancer Cells* 3: 347-350.
- Hemler ME (1990). VLA proteins in the integrin family. Structures, functions, and their role on leukocytes. *Annual Reviews in Immunology* 8, 365-400.

Hénon PR, Butturini A, Gale RP (1991). Blood-derived haematopoietic cell transplants: blood to blood? *Lancet* 337: 961- 963.

Hénon PR (1993). Peripheral blood stem cell transplants: past, present and future. *Stem Cells* 11: 154-172.

Herbein G, Sovalat H, Wunder E, Baerenzung M, Bachorz J, Lewandowski H, Schweitzer C, Schmitt C, Kirn A, Henon P (1994). Isolation and identification of two CD34+ cell subpopulations from normal human peripheral blood. *Stem cells* 12: 187-197.

Heslop HE, Duncombe AS, Reittie JE, Bello-Fernandez C, Gottlieb DJ, Prentice HG, Mehta AB, Hoffbrand AV, Brenner MK (1991). Interleukin 2 infusion induces haemopoietic growth factors and modifies marrow regeneration after chemotherapy or autologous marrow transplantation. *British Journal of Haematology* 77: 237-244.

Hoffman RA, Hansen WP (1981). Immunofluorescent analysis of blood cells by flow cytometry. *International Journal of Immunopharmacology* 3: 249-254.

Hogg N, Bennett R, Cabanas C, Dransfield I (1992). Leukocyte integrin activation. *Kidney International* 41: 613-616.

Holyoake TL, Freshney MG, Konwalinka G, Haun M, Petzer A, Fitzsimmons E, Lucie NP, Wright EG, Pragnell IB (1993). Mixed colony formation in vitro by the heterogeneous compartment of multipotential progenitors in human bone marrow. *Leukemia* 7: 207- 213.

Holyoake TL, Franklin IM (1994). Bone marrow transplants from peripheral blood. *British Medical Journal* 309: 4-5.

Holyoake TL, Alcorn MJ (1994). CD34+ haemopoietic cells: biology and clinical applications. *Blood Reviews* 8: 113-124.

Holzer H, Biehl J, Antin P, Takunaka S, Sasse J, Pacifici M, Holtzer S (1983). Quantal and proliferative cell cycles: how lineages generate cell diversity and maintain fidelity. *Progress in Clinical and Biological Research* 134: 213-227.

Huang S, Terstappen LWMM (1992). Formation of both haematopoietic microenvironment and hematopoietic stem cells from single human marrow stem cells. *Nature* 360: 745-749. Retraction: *Nature* 368: 664 (1994).

Huang S, Terstappen LWMM (1994). Lymphoid and myeloid differentiation of single human CD34<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>-</sup> hematopoietic stem cells. *Blood* 83: 1515-1526.

Humphries MJ (1990). The molecular basis and specificity of integrin-ligand interactions. *Journal of Cell Science* 97: 585-592.

Hurley R, McCarthy JB, Verfaillie CM (1993). Direct contact with marrow stroma has negative regulatory effects on hematopoietic progenitors. *Blood* 82: 71 *abstract*.

Hurwitz CA, Gore SD, Stone KD, Civin CI (1992). Flow cytometric detection of rare normal human marrow cells with immunophenotypes characteristic of acute lymphoblastic leukaemia cells. *Leukaemia* 6: 233-239.

Hutton RA, Warrell DA (1993). Action of snake venom components on the haemostatic system. *Blood Reviews* 1993; 7: 176-189.

Hynes RO (1987). Integrins: A family of cell surface receptors. *Cell* 48, 549-554.

Hynes RO (1990). *Fibronectins*. Springer Verlag, New York.

Hynes RO (1992a). Specificity of cell adhesion in development: the cadherin superfamily. *Current Opinion in Genetics and Development* 2: 621-624.

Hynes RO (1992b). Integrins: versatility, modulation and signaling in cell adhesion. *Cell* 69: 11-25.

Ichikawa Y, Pluznik DH, Sachs L (1966). In vitro control of the development of macrophage and granulocyte colonies. *Proceedings of the National Academy of Sciences USA*: 56: 488-495.

Irie S, Tavassoli M (1991). Transendothelial transport of macromolecules: the concept of tissue-blood barriers. *Cell Biology Reviews* 25: 317-333.

Issaad C, Croisille L, Katz A, Vainchenker W, Coulombel L (1993). A murine stromal cell line supports the proliferation of early human CD34<sup>+</sup>/CD38<sup>-</sup> progenitor cells in long-term cultures and short-term semisolid colony assays. *Blood* 81: 2916-2924.

Jalkanen S, Bargatze RF, de los Toyos J, Butcher EC (1987). Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal or synovial endothelial cells. *Journal of Cell Biology* 105: 983-990.

Jauregui HO, McMillan PN, Driscoll J, Naik S (1986). Attachment and long-term survival of adult rat hepatocytes in primary monolayer cultures: comparison of different substrata and tissue culture media formulations. *In Vitro Cellular and Developmental Biology* 22: 13-22.



- Jones RJ, Wagner JE, Celano P, Zicha MS, Sharkas SJ (1990). Separation of pluripotent hematopoietic stem cells from spleen colony-forming cells. *Nature* 347: 188-189.
- Kansas GS, Muirhead J, Dailey MO (1990). Expression of the CD11/CD18 leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. *Blood* 76: 2483-2492.
- Karakantza M, Gibson FM, Cavenagh JD, Ball SE, Gordon MY, Gordon-Smith EC (1994). SLe<sup>x</sup> expression of normal CD34 positive bone marrow haemopoietic progenitor cells. *British Journal of Haematology* 86: 883-886.
- Karlsson S (1991). Treatment of genetic defects in hematopoietic function by gene transfer. *Blood* 78: 2481-2492.
- Katz FE, Tindle R, Sutherland DR, Greaves MF (1985). Identification of a membrane glycoprotein associated with haemopoietic progenitor cells. *Leukaemia Research* 9: 191-198.
- Kay HEM (1965). How many generations? *Lancet* 2: 418-419.
- Keating A, Wight M, Kinsell M, Singer JW (1984). Proteoglycan synthesis by hematopoietic microenvironmental cells. *Clinical Research* 32: 311 *abstract*.
- Kincade PW (1987). Experimental models for understanding B lymphocyte formation. *Advances in Immunology* 41: 181-267.
- Kittler EL, McGrath H, Temeles D, Crittenden RB, Kister VK, Quesenberry PJ (1992). Biologic significance of constitutive and subliminal growth factor production by bone marrow stroma. *Blood* 79: 3168-178.
- Kobayashi M, Imamura M, Uede T, Sakurada K, Maeda S, Iwasaki H, Tsuda Y, Musashi M, Miyazaki T (1994). Expression of adhesion molecules on human hematopoietic progenitor cells at different maturational stages. *Stem Cells* 12: 316-321.
- Koeffler HP, Billing R, Lusa AJ, Sparkes R, Golde DW (1980). An undifferentiated variant derived from the human acute myelogenous leukaemia cell line (KG-1). *Blood* 56: 265-273.
- Koenigsmann M, Griffin JD, DiCarlo J, Cannistra SA (1992). Myeloid and erythroid progenitor cells from normal bone marrow adhere to collagen type I. *Blood* 79: 657-665.
- Kohler G, Milstein C (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495- 497.



Koller MR, Bender JG, Miller WM, Papoutsakis ET (1992). Reduced oxygen tension increases hematopoiesis in long-term cultures of human stem and progenitor cells from cord blood and bone marrow. *Experimental Hematology* 20: 264-270.

Koshiishi I, Shizari M, Underhill CB (1994). CD44 can mediate the adhesion of platelets to hyaluronan. *Blood* 84: 390-396.

Koury MJ (1992). Programmed cell death (apoptosis) in hematopoiesis. *Experimental Hematology* 20: 391-394.

Kuipers TW (1993). Terminal glycosyltransferase activity: a selective role in cell adhesion. *Blood* 81: 873-882.

Kurnick JE, Robinson WA (1971). Colony growth of human peripheral white blood cells in vitro. *Blood* 37: 136-141.

Lackie JM (1991). Adhesion from flow. *In: Curtis ASG, Lackie JM eds. Measuring Cell Adhesion.* Wiley, England.

Lajtha LG, Possi LV, Schofield R, Fox M (1969). Kinetic properties of haemopoietic stem cells. *Cell and Tissue Kinetics* 2: 39-49.

Lajtha LG (1980). The common ancestral stem cell. *In: Wintrobe MW ed. Blood, Pure and Eloquent.* McGraw-Hill, New York.

Lal RB, Edison LJ, Chused TM (1988). Fixation and long-term storage of human lymphocytes for surface marker analysis by flow cytometry. *Cytometry* 9: 213-219.

Lanier LI, Warner NL (1981). Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry analysis (FACS) analysis. *Journal of Immunological Methods* 47: 25-30.

Lansdorp PM, Dougherty GJ, Humphries RK (1989). CD34 epitopes. *In: Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr eds. Leucocyte Typing IV; White Cell Differentiation Antigens.* Oxford University Press, Oxford: 826-827.

Lansdorp PM, Sutherland HJ, Eaves CJ (1990). Selective expression of CD45 isoforms on functional subpopulations of CD34<sup>+</sup> hemopoietic cells from human bone marrow. *Journal of Experimental Medicine* 172: 363-366.

Lansdorp PM, Dragowska W (1992). Long-term erythropoiesis from constant numbers of CD34<sup>+</sup> cells in serum-free cultures initiated with highly purified progenitor cells from human bone marrow. *Journal of Experimental Medicine* 175: 1501-1509.

Lansdorp PM, Dragowska W, Mayani H (1993). Ontogeny-related changes in proliferative potential of human hematopoietic cells. *Journal of Experimental Medicine* 178: 787-791.

Lapidot T, Pflumio F, Doedens M, Murdoch B, Williams DE, Dick JE (1992). Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* 255: 1137-1141.

Lapidot T, Pflumio F, Dick JE (1993). Modeling human hematopoiesis in immunodeficient mice. *Laboratory Animal Science* 43: 147-150.

Larson RS, Springer TA (1990). Structure and function of leucocyte integrins. *Immunological Reviews* 114, 181-217.

Lasky LA (1991). Lectin cell adhesion molecules (LEC-CAMs): a new family of cell adhesion proteins involved in inflammation. *Journal of Cellular Biochemistry* 45: 139-146.

Lasky LA (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science* 258: 964-969.

Larrick JW, Burck KL (1991). *Gene therapy: application of molecular biology*. Elsevier: New York.

Lawrence MB, Springer TA (1991). Leukocytes role on a selectin at physiologic shear flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* 65: 859-873.

Leary AG, Ogawa M (1987). Blast cell colony assay for umbilical cord blood and adult bone marrow progenitors. *Blood* 69: 953-956.

Leary AG, Zeng HQ, Clark SC, Ogawa M (1992). Growth factor requirements for survival in  $G_0$  and entry into the cell cycle of primitive human hemopoietic progenitors. *Proceedings of the National Academy of Sciences USA* 89: 4013-4017.

Leavesley DI, Oliver JM, Swart BW, Berndt MC, Haylock DN, Simmons PJ (1994). Signals from platelet/endothelial cell adhesion molecule (PECAM-1, CD31) enhance the adhesive activity of the VLA-4 integrin of human CD34+ hematopoietic progenitor cells. *Journal of Immunology* 153: 4673-4683.

Lemischka IR, Raulet CH, Mulligan RC (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell* 45: 917-927.

Lerat H, Lissitzky JC, Singer JW, Keating A, Herve P, Charbord P (1993). Role of stromal cells and macrophages in fibronectin biosynthesis and matrix assembly in human long-term marrow cultures. *Blood* 82: 1480-1492.

- Lewandowska K, Balachander N, Sukenik CN, Culp LA (1989). Modulation of fibronectin adhesive functions for fibroblasts on neural cells by chemically characterized substrata. *Journal of Cellular Physiology* 141: 334-345.
- Lewinsohn DM, Nagler A, Ginzton N, Greenberg P, Butcher EC (1990). Haemopoietic progenitor cell expression of the H-CAM (CD44) homing-associated adhesion molecule. *Blood* 75: 589-595.
- Lewis L, Blackett NM, Gordon MY (1994). The kinetics of colony formation by CFU-GM *in vitro*. *British Journal of Haematology* 88: 440-442.
- Lichtman MA, Packman CH, Constone LS (1989). Molecular and cellular traffic across the marrow sinuses. In: Tavassoli M, ed. *Handbook of the Hemopoietic Microenvironment*. Humana Press, New Jersey: 87-140.
- Lichtman MA (1991). The ultrastructure of the hemopoietic environment of the marrow. *Experimental Hematology* 9: 391-410.
- Liesveld JL, Abboud CN, Duerst RE, Ryan DH, Brennan JK, Lichtman MA (1989). Characterisation of human marrow stromal cells: role in progenitor cell binding and granulopoiesis. *Blood* 73: 1794-1800.
- Liesveld JL, Winslow JM, Kempski MC, Ryan DH, Brennan JK, Abboud CN (1991). Adhesive interactions of normal and leukaemic human CD34<sup>+</sup> myeloid progenitors: role of marrow stromal, fibroblast and cytomatrix components. *Experimental Hematology* 19: 63-70.
- Liesveld JL, Winslow JM, Frediani KE, Ryan DH, Abboud CN (1993). Expression of integrins and examination of their adhesive function in normal and leukaemic hematopoietic cells. *Blood* 81: 112-121.
- List AF, Spier CM, Cline A, Doll DC, Garewal H, Morgan R, Sandberg AA (1991). Expression of the multidrug resistance gene product (P-glycoprotein) in myelodysplasia is associated with a stem cell phenotype. *British Journal of Haematology* 78: 28-34.
- Loken MR, Shah VO, Dattilio KL, Civin CI (1987a) Flow cytometric analysis of human bone marrow: I. Normal erythroid development. *Blood* 69: 255-263.
- Loken MR, Shah VO, Dattilio KL, Civin CI (1987b). Flow cytometric analysis of human bone marrow: II. Normal B lymphocyte development. *Blood* 70: 1316-1324.
- Loken MR, Brosnan JM, Bach BA, Ault KA (1990). Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 11: 453-459.

- Long MW, Dixit VM (1990). Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. *Blood* 75: 2311-2318.
- Long MW (1992). Blood cell cytoadhesion molecules. *Experimental Hematology* 20: 288-301.
- Long M, Briddell R, Walter AW, Bruno E, Hoffman R (1992). Human hematopoietic stem cell adherence to cytokines and matrix molecules. *Journal of Clinical Investigation* 90: 251-255.
- Louache F, Debili N, Marandin A, Coulombel L, Vainchenker W (1994). Expression of CD4 by human hematopoietic progenitors. *Blood* 84: 3344-3355.
- Lozzio CB, Lozzio BB (1975). Human chronic myelogenous leukaemia cell-line with positive Philadelphia chromosome. *Blood* 45: 321-334.
- Lum LG (1990). Recapitulation of immune ontogeny: a vital component for the success of bone marrow transplantation. *In: Champlin R ed. Bone Marrow Transplantation*. Kluwer Academic Publishers, New York: 27-54.
- Lund-Johansen F, Terstappen LW (1993). Differential surface expression of cell adhesion molecules during granulocyte maturation. *Journal of Leukocyte Biology* 54: 47-55.
- Magli MC, Iscove NN, Odartchenko N (1982). Transient nature of early haematopoietic spleen colonies. *Nature* 295: 527-529.
- Majdic O, Stöckl J, Pickl WF, Bohuslav J, Strobl H, Scheinecker C, Stockinger H, Knapp W (1994). Signaling and induction of enhanced cytoadhesiveness via hematopoietic progenitor cell surface molecule CD34. *Blood* 83: 1226-1234.
- Makgoba MW, Sanders ME, Luce GEG, Dustin ML, Springer TA, Clark EA, Mannoni P, Shaw S (1988). ICAM-1 (CD54): A ligand for LFA-1-dependent adhesion of B, T and myeloid cells. *Nature* 331: 86-88.
- Makgoba MW, Bernard A (1993). Cell adhesion in the hematopoietic system. *In: Brenner MK, Hoffbrand AV eds. Recent Advances in Haematology*. Churchill Livingstone, Edinburgh: 7: 85-99.
- Martini E, D'Hautcourt JL, Brando B, Lawry J, O'Connor JE, Sansonetty F (1990). First European Quality Control of Cellular Phenotyping by Flow Cytometry 1989. Frisons-Roch, Paris.
- Masek LC, Sweetenham JW (1994). Isolation and culture of endothelial cells from human bone marrow. *British Journal of Haematology* 88: 855-865.

- Masek LC, Sweetenham JW, Whitehouse JMA, Schumacher U (1994). Immunolectin and enzyme-histochemical characterisation of human bone marrow endothelium. *Experimental Hematology* 22: 1203-1209.
- Masumoto A, Hemler ME (1993). Multiple activation states of VLA- 4. Mechanistic differences between adhesion to CS1 / fibronectin and vascular cell adhesion molecule-1. *Journal of Biological Chemistry* 268: 228-234.
- Matsumura Y, Tarin D (1992). Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 340: 1053-1058.
- Mauch P, Greenberger JS, Botnick L, Hannon E, Hellman S (1980). Evidence for structural variation in self-renewal capacity within long-term bone marrow cultures. *Proceedings of the National Academy of Sciences USA* 77: 2927-2930.
- McCulloch EA (1985). Normal stem cells and the clonal hemopathies. *In*: Cronkite EP, Dainiak N, McCaffrey RP, Palek J, Quesenberry PJ *eds*. Hematopoietic Stem Cell Physiology. Progress in Clinical and Biological Research. Alan R Liss Inc, New York 184: 21-38.
- McCulloch EA (1987). Lineage infidelity or lineage promiscuity? *Leukaemia* 1: 235.
- McNiece IK, Stewart FM, Deacon DM, Temeles DS, Zsebo KM, Clark SC, Quesenberry PJ (1989). Detection of a human CFC with a high proliferative potential. *Blood* 74: 609-612.
- Medvinsky AL, Samoylina NL, Muller AM, Dzierzak EA (1993). An early pre-liver intraembryonic source of CFU-S in the developing mouse. *Nature* 364: 64-69.
- Metcalf D, Warner NL, Nossal GJV, Miller JFAP, Shortman K, Rabellino E (1975). Growth of B lymphocyte colonies in vitro from mouse lymphoid organs. *Nature* 255: 630-631.
- Metcalf D, MacDonald HP, Odartchenko N, Sordat B (1975). Growth of mouse megakaryocyte colonies *Proceedings of the National Academy of Sciences USA* 72: 1744-1748.
- Metcalf D (1993). Hematopoietic regulators: redundancy or subtlety? *Blood* 82: 3515-3523.
- Miller AD (1990). Progress toward human gene therapy. *Blood* 76: 271-278.
- Miltenyi S, Muller W, Weichel W, Radbruch A (1990). High gradient magnetic cell separation with MACS. *Cytometry* 11: 231-238.



Minguell JJ, Hardy CL, Tavassoli M (1992). Membrane-associated chondroitin sulphate proteoglycan and fibronectin mediate the binding of hemopoietic progenitor cells to stromal cells. *Experimental Cell Research* 201: 200-207.

Minguell JJ, Hardy CL, Tavassoli M (1993). Adhesive interaction of hemopoietic progenitor cell membrane with the RGD domain of fibronectin. *Biochimica et Biophysica Acta* 1151: 120-126.

Miyake K, Medina KL, Hayashi SI, Ono S, Hamaoka T, Kincade PW (1990). Monoclonal antibodies to Pgp/CD44 block lympho-hemopoiesis in long-term bone marrow cultures. *Journal of Experimental Medicine* 171: 477-488.

Miyake K, Underhill CB, Lesley J, Kincade PW (1990). Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *Journal of Experimental Medicine* 172: 69-75.

Miyake K, Weissman IL, Greenberger JS, Kincade PW (1991). Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *Journal of Experimental Medicine* 173: 599-607.

Miyake K, Medina K, Ishihara K, Kimoto M, Auerbach R, Kincade PW (1991). A VCAM-like adhesion molecule on murine bone marrow stromal cells mediates binding of lymphocyte precursors in culture. *Journal of Cell Biology* 114: 557-565.

Miyake K, Hasunuma Y, Yagita H, Kimoto M (1992). Requirement for VLA-4 and VLA-5 integrins in lymphoma cells binding to and migration beneath stromal cells in culture. *Journal of Cell Biology* 119: 653-662.

Mobley JL, Ennis E, Schimizu Y (1994). Differential activation- dependent regulation of integrin function in cultured human T- leukaemic cell lines. *Blood* 83: 1039-1050.

Möhle R, Haas R, Hunstein W (1993). Expression of adhesion molecules and c-kit on CD34<sup>+</sup> hematopoietic progenitor cells: comparison of cytokine-mobilized blood stem cells with normal bone marrow and peripheral blood. *Journal of Hematotherapy* 2: 483-489.

Molgaard H, Brown J, Simmons D, Spurr N, Healy L, Greaves M (1989). Molecular characterisation of human and murine CD34. *In*: Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr, eds. *Leucocyte Typing IV; White Cell Differentiation Antigens*. Oxford University Press, Oxford: 827-829.

Moore MAS (1991). Clinical implications of positive and negative hematopoietic stem cell regulators. *Blood* 78: 1-19.

Moore MAS (1992). Does stem cell exhaustion result from combining haematopoietic growth factors with chemotherapy? If so, how do we prevent it? *Blood* 80: 3-7.

Moore T, Huang S, Terstappen LW, Bennett M, Kumar V (1994). Expression of CD43 on murine and human pluripotential hematopoietic stem cells. *Journal of Immunology* 153: 4978-4987.

Moreau I, Andreoni C, Caux C, Saeland S, Rigal D (1992). Modification of human long-term bone marrow cultures: establishment of a functional stromal microenvironment devoid of myeloid progenitors. *European Journal of Haematology* 49: 29-35.

Morimoto K, Robin E, Le Bousse-Kerdiles MC, Li Y, Clay D, Jasmin C, Smadja-Joffe F (1994). CD44 mediates hyaluronan binding by human myeloid KG1a and KG1 cells. *Blood* 83: 657-662.

Morris AJ, Turnbull JE, Riley GP, Gordon MY, Gallagher JT (1991). Production of heparan sulphate proteoglycans by human bone marrow stromal cells. *Journal of Cell Science* 99: 149-155.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65: 55-63.

Müller-Sieburg CE, Townsend K, Weissman IL, Rennick D (1988). Proliferation and differentiation of highly enriched mouse hematopoietic stem cells and progenitor cells in response to defined growth factors. *Journal of Experimental Medicine* 167: 1825-1840.

Nakahata T, Gross AJ, Ogawa M (1982). A stochastic model of self-renewal and commitment to differentiation of the primitive hemopoietic stem cells in culture. *Journal of Cell Physiology* 113: 455-458.

Nakahata T, Ogawa M (1982). Identification in culture of a class of hematopoietic colony-forming units with extensive capability to self-renew and generate multipotential hematopoietic colonies. *Proceedings of the National Academy of Sciences USA* 79: 3843-3847.

Narayanan N, Su N, Bedard P (1991). Inhibitory and stimulatory effects of fluoride on the calcium pump of cardiac sarcoplasmic reticulum. *Biochimica et Biophysica Acta* 1070: 83-91.

Nathan C, Sporn M (1991). Cytokines in context. *Journal of Cell Biology* 113: 981-986.

Neben S, Redfearn WJ, Parra M, Brecher G, Pallavicini MG (1991). Short- and long-term repopulation of lethally irradiated mice by bone marrow stem cells enriched on the basis of light scatter and Hoechst 33342 fluorescence. *Experimental Hematology* 19: 958-967.

Nicol AJ, Hows JM, Bradley BA (1994). Cord blood transplantation: a practical option? *British Journal of Haematology* 87: 1-5.

Nojima Y, Humphries MJ, Mould AP, Komoriya A, Yamada KM, Schlossman SF, Morimoto C (1990). VLA-4 mediates CD3-dependent CD4<sup>+</sup> T cell activation via the CS1 alternatively spliced domain of fibronectin. *Journal of Experimental Medicine* 172: 1185-1192.

Nordon RE, Milthorpe BK, Schindhelm K, Slowiaczek PR (1994). An experimental model of affinity cell separation. *Cytometry* 16: 25- 33.

Ogawa M, Porter PN, Nakahata T (1983). Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review). *Blood* 61: 823-829.

Ogawa M (1993). Differentiation and proliferation of hematopoietic stem cells. *Blood* 81: 2844-2853.

Okumura N, Tsuji K, Nakahata T (1992). Changes in cell surface antigen expression during proliferation and differentiation of human erythroid progenitors. *Blood* 80: 642-650.

Pantel K, Nakeff A (1993) The role of lymphoid cells in hematopoietic regulation. *Experimental Hematology* 21: 738-742.

Papayannopoulou T, Brice M, Broudy VC, Zsebo KM (1991). Isolation of c-kit receptor-expressing cells from bone marrow, peripheral blood, and fetal liver: functional properties and composite antigenic profile. *Blood* 78: 1403-1412.

Papayannopoulou T, Brice M (1992). Integrin expression profiles during erythroid differentiation. *Blood* 79: 1686-1694.

Papayannopoulou T, Nakamoto B (1993). Peripheralization of hemopoietic progenitors in primates treated with anti-VLA-4 integrin. *Proceedings of the National Academy of Sciences USA* 90: 9374-9378.

Pappenheim A (1898). Abstammung und entstehung der rothen blutzelle. *Archiv fur pathologische Anatomie und Physiologie, und fur klinische Medecin*. Berlin.151: 89-158.

Parish CR, Hogarth PM, McKenzie IF (1988). Evidence that Thy-1 and Ly-5 (T-200) antigens interact with sulphated carbohydrates. *Immunology and Cell Biology* 66: 221-230.

Parkman R (1986). The application of bone marrow transplantation to the treatment of genetic diseases. *Science* 232: 1373-1378.

Parks DR, Lanier LL, Herzenberg LA (1986). Flow Cytometry and fluorescence activated cell sorting. *In*: Weir DM, Herzenberg LA, Blackwell C, Herzenberg LA eds. *Handbook of Experimental Immunology*. Vol 1. Immunochemistry. 4th Ed. Blackwell Scientific Publications, Oxford: 29.1-29.21.

Patel VP, Lodish HF (1986). The fibronectin receptor on mammalian erythroid precursor cells: Characterization and developmental regulation. *Journal of Cell Biology* 102: 449-456.

Peschel CH, Köller U (1989). Cluster Report: CD34. *In*: Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr eds. *Leucocyte Typing IV; White Cell Differentiation Antigens*. Oxford University Press, Oxford: 817-818.

Peschel C, Paul WE, Ohara J, Green I (1987). Effects of B cell stimulatory factor -1 / interleukin 4 on hematopoietic progenitor cells. *Blood* 70: 254-263.

Picker LJ, Warnock RA, Burns AR, Doerschuk CM, Berg EL, Butcher EC (1991). The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* 66: 921-933.

Piersbacher MD, Ruoslahti E (1984). The cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* 309: 30-33.

Piersbacher MD, Ruoslahti E (1987). Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *Journal of Biological Chemistry* 262: 17294-17298.

Ploemacher RE, Brons RHC (1989). Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence of a pre-CFU-S cell. *Experimental Hematology* 17: 263-266.

Pluznick PH, Sachs L (1965). The cloning of normal "mast" cells in tissue culture. *Journal of Cell Physiology* 66: 319-324.

Potten CS, Loeffler M (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the Crypt. *Development* 110: 1001-1020.

Quesenberry PJ, Temeles D, McGrath H, Lowry P, Meyer D, Kittler E, Deacon D, Kister K, Crittenden R, Srikumar K (1991). Long-term marrow cultures: human and murine systems. *Journal of Cellular Biochemistry* 45: 273-278.

Quesenberry PJ (1991). The blueness of stem cells. *Experimental Hematology* 19: 725-728.

Rafii S, Shapiro F, Rimarachin J, Nachman RL, Ferris B, Weksler B, Moore MA, Asch AS (1994). Isolation and characterization of human bone marrow microvascular endothelial cells: hematopoietic progenitor cell adhesion. *Blood* 84: 10-19.

Rahilly MA, Fleming S (1993). The specificity of integrin-ligand interactions in cultured human renal epithelium. *Journal of Pathology* 170: 297-303.

Ravagnani F, Siena S, Bregni M, Belli N, Lansdorp PM, Notti P, Pellegris G, Gianni AM (1991). Methodologies to estimate circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Haematologica* 76: 46-49.

Reisbach G, Bartke I, Kempkes B, Kostka G, Ellwart J, Birner A, Thalmeier K, Mailhammer R, Bornkamm GW, Ullrich A, Dörmer P (1993). Characterization of hemopoietic cell populations from human cord blood expressing c-kit. *Experimental Hematology* 21: 74-79.

Reuss-Borst MA, Buhring HJ, Klein G, Muller CA (1992). Adhesion molecules on CD34+ hematopoietic cells in normal human bone marrow and leukaemia. *Annals of Hematology* 65: 169-174.

Rice A, Boiron JM, Barbot C, Dupouy M, Dubosc-Marchenay N, Dumain P, Lacombe F, Reiffers J (1995). Cytokine mediated expansion of 5-FU resistant peripheral blood stem cells. *Experimental Hematology* 23: 303-308.

Riley GP, Gordon MY (1987). Characterization of cultured stromal layers derived from fetal and adult hemopoietic tissues. *Experimental Hematology* 15: 78-84.

Rill DR, Santana VM, Roberts WM, Nilson T, Bowman LC, Krance RA, Heslop HE, Moen RC, Ihle JN, Brenner MK (1994). Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells. *Blood* 84: 380-383.

Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM (1988). Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haemopoiesis. *Nature* 332: 376-378.



- Roscoe RA, Rybka WB, Winkelstein A, Houston AM, Kiss JE (1994). Enumeration of CD34+ hematopoietic stem cells for reconstitution following myeloablative therapy. *Cytometry* 16: 74-79.
- Roseblatt M, Vuillet-Gaugler MH, Leroy C, Coulombel L (1991). Coexpression of two fibronectin receptors VLA-4 and VLA-5 by human erythroblastic precursor cells. *Journal of Clinical Investigation* 87: 6-11.
- Rosen SD (1990). The LEC-CAMs: an emerging family of cell-cell adhesion receptors based on carbohydrate recognition. *American Journal of Respiratory Cellular and Molecular Biology* 3: 397-402.
- Rowley SD, Sharkis SJ, Hattenburg C, Sensenbrenner LL (1987). Culture from human bone marrow of blast cell progenitor cells with an extensive proliferative capacity. *Blood* 69: 804-808.
- Ruoslahti E, Pierschbacher MD (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell* 44: 517-518.
- Ruoslahti E, Pierschbacher MD (1987). New perspectives in cell adhesion: RGD and integrins. *Science* 238: 491-497.
- Ruoslahti E, Yamaguchi Y (1991). Proteoglycans as modulators of growth factor activity. *Cell* 64: 867-869.
- Ruoslahti E, Reed JC (1994). Anchorage dependence, integrins and apoptosis. *Cell* 77: 477-478.
- Ryan D, Kossover S, Mitchell S, Frantz C, Hennessy L, Cohen H (1986). Subpopulations of common acute lymphoblastic leukaemia antigen-positive lymphoid cells in normal bone marrow identified by hematopoietic differentiation antigens. *Blood* 68: 417-425.
- Saeland S, Duvert V, Caux C, Pandrau D, Favre C, Vallé A, Durand I, Charbord P, de Vries J, Banchereau J (1992). Distribution of surface-membrane molecules on bone marrow and cord blood CD34+ hematopoietic cells. *Experimental Hematology* 20: 24-33.
- Sangster RN, Minowada J, Suci-Foca N, Minden M, Mak TW (1986). Rearrangement and expression of the alpha, beta and gamma chain T cell receptor genes in human thymic leukaemic cells and functional T cells. *Journal of Experimental Medicine* 163: 1491-1508.

- Säuberlich S, Kisch A, Serke S (1994). Determination of CD34<sup>+</sup> hemopoietic cells by multiparameter flow cytometry: technical remarks. *In*: Wunder E, Sovalat H, Hénon PR, Serke S eds. Hematopoietic Stem Cells: The Mulhouse Manual. AlphaMed Press, Dayton, Ohio. 45-60.
- Saunders S, Bernfield M (1988). Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. *Journal of Cell Biology* 106: 423-430.
- Schwarzbauer JE (1991). Fibronectin: from gene to protein. *Current Opinion in Cell Biology* 3: 786-791.
- Schwarzbauer JE (1991). Alternative splicing of fibronectin: Three variants, three functions. *Bioessays* 13: 527-533.
- Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI (1992). Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proceedings of the National Academy of Sciences USA* 89: 12160-4.
- Serke S, Säuberlich S, Huhn D (1991). Multiparameter flow-cytometrical quantitation of circulating CD34<sup>+</sup>-cells: correlation to the quantitation of circulating haemopoietic progenitor cells by in vitro colony-assay. *British Journal of Haematology* 1991; 77: 453-459.
- Serke S, Säuberlich S, Abe Y, Huhn D (1991). Analysis of CD34-positive hemopoietic progenitor cells from normal human adult peripheral blood: flow cytometrical studies and in vitro colony (CFU-GM, BFU-E) assays. *Annals of Hematology* 62: 45-53.
- Shah VO, Civin CI, Loken MR (1988). Flow cytometric analysis of human bone marrow IV. Differential quantitative expression of T-200 common leukocyte antigen during normal hemopoiesis. *Journal of Immunology* 140: 1861-1867.
- Shirota T, Tavassoli M (1991). Cyclophosphamide-induced alterations of bone marrow endothelium: implications in homing of marrow cells after transplantation. *Experimental Hematology* 19: 369-373.
- Shirota T, Tavassoli M (1992). Alterations of bone marrow sinus endothelium induced by ionizing radiation: implications in the homing of intravenously transplanted marrow cells. *Blood Cells* 18: 197-214.
- Shpall EJ, Jones RB, Bearman SI, Purdy MH, Franklin WA, Heimfeld S, Berenson RJ (1994). Transplantation of CD34<sup>+</sup> hematopoietic progenitor cells. *Journal of Hematotherapy* 3: 145-147.

Siczkowski M, Andrew T, Amos S, Gordon MY (1993). Hyaluronic acid regulates the function and distribution of sulphated glycosaminoglycans in bone marrow stromal cultures. *Experimental Hematology* 21: 126-130.

Siczkowski M, Clarke D, Gordon MY (1992). Binding of primitive hematopoietic progenitor cells to marrow stromal cells involves heparan sulphate. *Blood* 80: 912-919.

Siena S, Bregni M, Brando B, Ravagnani F, Bonadonna G, Gianni AM (1989). Circulation of CD34-positive hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide treated patients: enhancement by intravenous recombinant granulocyte-macrophage colony-stimulating factor. *Blood* 74: 1905-1914.

Siena S, Bregni M, Brando B, Belli N, Ravagnani F, Gandola L, Stern AC, Lansdorp PM, Bonadonna G, Gianni AM (1991a). Flow cytometry for the clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood*; 77: 400-409.

Siena S, Bregni M, Brando B, Belli N, Lansdorp PM, Bonadonna G, Gianni AM (1991b). Flow cytometry to estimate circulating hematopoietic progenitors for autologous transplantation: comparative analysis of different CD34 monoclonal antibodies. *Haematologica* 76: 330-333.

Siena S, Bregni M, Belli N, Ravagnani F, Notti P, Magni M, Di Nicola M, Bonadonna G, Gianni AM (1992). Practical aspects of flow cytometry to guide large-scale collection of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *International Journal of Cell Cloning* 10 (Suppl 1): 26-29 .

Siena S, Bregni M, Gianni AM (1993). Estimation of peripheral blood CD34+ cells for autologous transplantation in cancer patients. *Experimental Hematology* 21: 203-205.

Siena S, Bregni M, Di Nicola M, Peccatori F, Magni M, Brando B, Ravagnani F, Gianni AM (1994). Milan protocol for clinical CD34<sup>+</sup> estimation in peripheral blood for autografting in patients with cancer. *In*: Wunder E, Sovalat H, Hénon PR, Serke S *eds*. *Hematopoietic Stem Cells: The Mulhouse Manual*. AlphaMed Press, Ohio: 23-30.

Silvestri F, Wunder E, Sovalat H, Henon P, Serke S (1993). Positive selection of CD34<sup>+</sup> cells: a short review of the immunoabsorption methods currently available for experimental and clinical use. Report on the 2nd European Workshop on Stem Cell Methodology, May 3-7 1993. *Journal of Hematotherapy* 2: 473-481.

Simmons PJ, Torok-Storb B (1991a). CD34 expression by stromal precursors in normal human adult bone marrow. *Blood* 78: 2848-2853.

Simmons PJ, Torok-Storb B, (1991b). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody. STRO-1. *Blood* 78: 55-62.

Simmons PJ, Masinovsky B, Longenecker BM, Berenson R, Torok-Storb B, Gallatin WM (1992). Vascular cell adhesion molecule-1 expressed by bone marrow stromal cells mediates the binding of hematopoietic progenitor cells. *Blood* 80: 388-395.

Simmons PJ, Niutta S, Ashman LK, Berndt MC, Juttner CA (1992). Primitive human bone marrow progenitors express L-Selectin and PECAM-1 (CD31). *Experimental Hematology* 20: 829 *abstract*.

Simmons PJ, Aylett GW, Niutta S, To LB, Juttner CA, Ashman LK (1994). c-kit is expressed by primitive human hematopoietic cells that give rise to colony-forming cells in stroma-dependent or cytokine-supplemented culture. *Experimental Hematology* 22: 157-165.

Singer JW, Keating A, Wight TN (1985). The human haemopoietic microenvironment. *In: Hoffbrand AV ed. Recent Advances in Haematology*. Churchill Livingstone, Edinburgh. 4: 1-24.

Smeland EB, Funderud S, Kvalheim G, Gaudernack G, Rasmussen AM, Rusten L, Wang MY, Tindle RW, Blomhoff HK, Egeland T (1992). Isolation and characterization of human hematopoietic progenitor cells: an effective method for positive selection of CD34+ cells. *Leukaemia* 6: 845-852.

Smith C, Gasparetto C, Collins N, Gillio A, Muench MO, O'Reilly RJ, Moore MAS (1991). Purification and partial characterisation of a human hematopoietic precursor population. *Blood*; 77: 2122-2128.

Soligo D, Schiró R, Luksch R, Manara G, Quirici N, Parravicini C, Deliliers GL (1990). Expression of integrins in human bone marrow. *British Journal of Haematology* 76: 323-332.

Sovalat H, Wunder E, Tienhaara A, Olofsson T, Fritsch G, Silvestri F, Serke S (1993). Commentary: prospects for standardization of stem cell determination within Europe. *Journal of Hematotherapy* 2: 293-296.

Sovalat H, Wunder E, Zimmermann R, Serke S (1994). Multicentric determination of CD34+ cells. *In: Wunder E, Sovalat H, Hénon PR, Serke S eds. Hematopoietic Stem Cells: The Mulhouse Manual*. AlphaMed Press, Ohio: 61-66.



Spangrude GL (1991). Hematopoietic stem cell differentiation. *Current Opinion in Immunology* 3: 171-178.

Spooncer E, Gallagher JT, Krizsa F, Dexter TM (1983). Regulation of haemopoiesis in long-term bone marrow cultures. IV. Glycosaminoglycan synthesis and stimulation of haemopoiesis by  $\beta$ -D-xylosides. *Journal of Cell Biology* 96: 510-514.

Springer TA, Lasky LA (1991). Cell adhesion. Sticky sugars for selectins (news). *Nature* 349: 196-197.

Springer TA (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76: 301-314.

Sredni B, Kalechman Y, Michlin H, Rozenszajn LA (1976). Development of colonies in vitro of mitogen stimulated mouse T lymphocytes. *Nature* 259: 130-132.

Srour EF, Brandt JE, Briddell RA, Leemhuis T, van Besien K, Hoffman R (1991). Human CD34<sup>+</sup> HLA-DR<sup>-</sup> bone marrow cells contain progenitor cells capable of self-renewal, multilineage differentiation, and long-term in vitro hematopoiesis. *Blood Cells* 17: 287-295.

Srour EF, Hoffman R, Zanjani D (1992). Animal models for human hematopoiesis. *Journal of Hematotherapy* 1: 143-153.

Srour EF (1994). Hematopoietic growth factors and transendothelial migration. *Experimental Hematology* 22: 237-238.

Stamenkovic I, Aruffo A, Amiot M, Seed B (1991). The hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO Journal* 10: 343-348.

Stelzer GT, Shults KE, Loken MR (1993). CD45 gating for routine flow cytometric analysis of human bone marrow specimens. *Annals of the New York Academy of Sciences*. 677: 265-280.

Stephenson JR, Axelrad AA, Mcleod DL, Shreeve MM (1971). Induction of colonies of haemoglobin synthesizing cells by erythropoietin in vitro. *Proceedings of the National Academy of Sciences USA*, 68: 1542-1546.

Strobl H, Takimoto M, Majdic O, Hocker P, Knapp W (1992). Antigenic analysis of human haemopoietic progenitor cells expressing the growth factor receptor c-kit. *British Journal of Haematology* 82: 287-294.

Strobl H, Takimoto M, Majdic O, Fritsch G, Scheinecker C, Hocker P, Knapp W (1993). Myeloperoxidase expression in CD34<sup>+</sup> normal human hematopoietic cells. *Blood* 82: 2069-2078.



Sutherland DR, Keating A (1992). The CD34 antigen: structure, biology and potential clinical applications. *Journal of Hematotherapy* 1: 115-129.

Sutherland DR, Marsh JC, Davidson J, Baker MA, Keating A, Mellors A (1992). Differential sensitivity of CD34 epitopes to cleavage by *Pasteurella haemolytica* glycoprotease: implications for purification of CD34-positive progenitor cells. *Experimental Hematology* 20: 590-599.

Sutherland DR, Keating A, Nayar R, Anania S, Stewart AK (1994). Sensitive detection and enumeration of CD34+ cells in peripheral and cord blood by flow cytometry. *Experimental Hematology* 22: 1003-1010.

Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM (1989). Characterization and partial purification of human marrow cells capable of initiating long term hematopoiesis in vitro. *Blood* 74: 1563-1570.

Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *Proceedings of the National Academy of Sciences USA* 87: 3584-3588.

Sutherland HJ, Eaves CJ, Lansdorp PM, Thacker JD, Hogge DE (1991). Differential regulation of primitive human hematopoietic cells in long-term cultures maintained on genetically engineered murine stromal cells. *Blood* 78: 666-672.

Takeichi M (1988). The Cadherins: Cell-cell adhesion molecules controlling animal morphogenesis. *Development* 102, 639-655.

Takeichi M (1990). Cadherins: a molecular family important in selective cell-cell adhesion. *Annual Reviews in Biochemistry* 59, 237-252.

Tanaka Y, Albelda SM, Horgan KJ, van Seventer GA, Shimizu Y, Newman W, Hallam J, Newman PJ, Buck CA, Shaw S (1992). CD31 expressed on distinctive T cell subsets is a preferential amplifier of beta 1 integrin-mediated adhesion. *Journal of Experimental Medicine* 176: 245-253.

Tanaka Y, Shaw S (1992). T cell adhesion cascades: general considerations and illustration with CD31. *Advances in Experimental Medicine and Biology* 323: 157-162.

Tavassoli M (1989). Fatty involution of marrow and the role of adipose tissue in hemopoiesis. In: Tavassoli M *ed.* *Handbook of the Hemopoietic Microenvironment*. New Jersey: Humana Press: 157-187.

Tavassoli M, Hardy C (1990). Molecular basis of homing of intravenously transplanted stem cells to the marrow. *Blood* 76: 1059- 1070.

Tavassoli M, Hardy C, Aizawa E, Matsuoka T, Minguell J (1990). Molecular mechanism of hematopoietic stem cell binding to the supportive stroma. *Progress in Clinical and Biological Research* 352: 87-95.

Tavassoli M (1991). Embryonic and fetal hemopoiesis: an overview. *Blood Cells* 17: 269-281.

Tavassoli M, Minguell JJ (1991). Homing of hemopoietic progenitor cells to the marrow. *Proceedings of the Society for Experimental Biology and Medicine* 196: 367-373.

Tavassoli M (1992). The role of conditioning regimens in homing of transplanted hemopoietic cells. *Bone Marrow Transplantation*; 10: 15-17.

Tavassoli M (1994). Embryonic origin of hematopoietic stem cells. *Experimental Hematology* 22: 7.

Teixido J, Hemler ME, Greenberger JS, Anklesaria M (1992). Role of  $\beta_1$  and  $\beta_2$  integrins in the adhesion of human CD34<sup>hi</sup> stem cells to bone marrow stroma. *Journal of Clinical Investigation* 90: 358-367.

Terstappen LWMM, Hollander Z, Meiners H, Loken MR (1990). Quantitative comparison of myeloid antigens on five lineages of mature peripheral blood cells. *Journal of Leukocyte Biology* 48: 138-148.

Terstappen LWMM, Loken MR (1990). Myeloid cell differentiation in normal bone marrow and acute myeloid leukaemia assessed by multidimensional flow cytometry. *Analytical Cellular Pathology* 2: 229-240.

Terstappen LWMM, Mickaels RA, Dost R, Loken MR (1990). Increased light scattering resolution facilitates multidimensional flow cytometric analysis. *Cytometry* 11: 506-512.

Terstappen LWMM, Safford M, Loken MR (1990). Flow cytometric analysis of human bone marrow: III. Neutrophil development. *Leukemia* 4: 657-663.

Terstappen LWMM, Huang S, Safford M, Lansdorp PM, Loken MR (1991). Sequential generations of hematopoietic colonies derived from single non lineage-committed CD34<sup>+</sup> CD38<sup>-</sup> progenitor cells. *Blood* 77: 1218-1227.

Terstappen LWMM, Huang S, Picker LJ (1992). Flow cytometric assessment of human T-cell differentiation in thymus and bone marrow. *Blood* 79: 666-677.

Terstappen LWMM, Buescher S, Nguyen M, Reading C (1992). Differentiation and maturation of growth factor expanded early hematopoietic progenitors assessed by multidimensional flow cytometry. *Leukemia* 6: 1001-1010.

Thomas ED (1994). The Nobel Lectures in Immunology. The Nobel Prize for Physiology or Medicine, 1990. Bone marrow transplantation - past, present and future. *Scandinavian Journal of Immunology* 39: 339-345.

Thomas ML (1989). The leukocyte common antigen family. *Annual Review of Immunology* 7: 339-369.

Thompson DW (1961). *On Growth and Form* (abridged edition). University Press, Cambridge.

Till JE, McCulloch EA (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Research* 14: 213-222.

To LB, Haylock DN, Dowse T, Simmons PJ, Trimboli S, Ashman LK, Juttner CA (1994). A comparative study of the phenotype and proliferative capacity of peripheral blood (PB) CD34+ cells mobilized by four different protocols and those of steady-phase PB and bone marrow CD34+ cells. *Blood* 84: 2930-2939.

Toksoz D, Dexter TM, Lord BI, Wright EG, Lajtha LG (1980). The regulation of haemopoiesis in long-term bone marrow cultures, II. Stimulation and inhibition of stem cell proliferation. *Blood* 55: 931-936.

Torok-Storb B (1988). Cellular interactions. *Blood* 72: 373-385.

Touraine JL, Raudrant D, Royo C, Rebaud A, Barbier F, Roncarolo MG, Touraine F, Laplace S, Gebuhrer L, Betvel H (1991). In utero transplantation of hemopoietic stem cells in humans. *Transplantation Proceedings* 23: 1706-1708.

Touw I, Lowenberg B (1983). No stimulative effect of adipocytes on hematopoiesis in long-term human bone marrow cultures. *Blood* 61: 770-774.

Traore Y, Hirn J (1994). Certain anti-CD34 monoclonal antibodies induce homotypic adhesion of leukaemic cell lines in a CD18-dependent and a CD18-independent way. *European Journal of Immunology* 24: 2304-2311.

Trentin JJ (1971). Determination of bone marrow stem cell differentiation by stromal hemopoietic inductive microenvironments (HIM). *American Journal of Pathology* 65: 621-628.

Trentin JJ (1989). Hemopoietic microenvironments: historical perspectives, status and projections. *In: Tavassoli M ed. Handbook of the Hemopoietic Microenvironment*. Humana Press, New Jersey: 1-86.

Tressel T, McCarthy JB, Calaycay J, Lee TD, Legesse K, Shively JE, Pande H (1991). Human plasma fibronectin. Demonstration of structural differences between the A and B chains in the CSIII region. *Biochemical Journal* 274: 731-738.

Trischmann TM, Schepers KG, Civin CI (1993). Measurement of CD34+ cells in bone marrow by flow cytometry. *Journal of Hematotherapy* 2: 305-313.

Tsai S, Patel V, Beaumont E, Lodish HF, Nathan DG, Sieff CA (1987). Differential binding of erythroid and myeloid progenitors to fibroblasts and fibronectin. *Blood* 69: 1587-1594.

Uchiyama H, Barut BA, Chauhan D, Cannistra SA, Anderson KC (1992). Characterization of adhesion molecules on human myeloma cell lines. *Blood* 80: 2306-2314.

Udomsakdi C, Eaves CJ, Sutherland HJ, Lansdorp PM (1991). Separation of functionally distinct subpopulations of primitive human hematopoietic cells using rhodamine 123. *Experimental Hematology* 19: 338-342.

Uhlman DL, Verfaillie C, Jones RB, Luikart SD (1991). BCNU treatment of marrow stromal monolayers reversibly alters haematopoiesis. *British Journal of Haematology* 78: 304-309.

Underhill CB (1992). The hyaluronan receptor. *Journal of Cell Science* 103: 293-298.

van Zant G, Goldwasser E (1979). Competition between erythropoietin and colony stimulating factor for target cells in mouse marrow. *Blood* 53: 946-965.

Verfaillie C, Blakolmer K, McGlave P (1990). Purified primitive human haematopoietic progenitor cells with long-term in vitro repopulating capacity adhere selectively to irradiated bone marrow stroma. *Journal of Experimental Medicine* 172: 509-512.

Verfaillie CM, McCarthy JB, McGlave PB (1990). Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alterations in their interaction with fibronectin. *Journal of Experimental Medicine* 174: 693-703.

Verfaillie CM (1992). Direct contact between human primitive hematopoietic progenitors and bone marrow stroma is not required for long-term in vitro hematopoiesis. *Blood* 79: 2821-2826.

Verfaillie CM, McCarthy JB, McGlave PB (1992). Mechanisms underlying abnormal trafficking of malignant progenitors in chronic myelogenous leukaemia: decreased adhesion to stroma and fibronectin but increased adhesion to the basement membrane components laminin and collagen type IV. *Journal of Clinical Investigation* 90: 1232-1241.



- Verfaillie CM (1994). Can human hematopoietic stem cells be cultured ex vivo? *Stem Cells* 12: 466-476.
- Verfaillie CM, Benis A, Iida J, McGlave PB, McCarthy JB (1994). Adhesion of committed human hematopoietic progenitors to synthetic peptides from the C-terminal heparin-binding domain of fibronectin: cooperation between the integrin  $\alpha 4\beta 1$  and the CD44 adhesion receptor. *Blood* 84: 1802-1811.
- Visser JWM, Van Bekkum DW. Purification of pluripotent hemopoietic stem cells: past and present. *Experimental Hematology* 1990; 18: 248-256.
- von Andrian UH, Chambers JD, Berg EL, Michie SA, Brown DA, Karolak D, Ramezani L, Berger EM, Arfors KE, Butcher EC. L-Selectin mediates neutrophil rolling in inflamed venules through sialyl Lewis<sup>x</sup>-dependent and -independent recognition pathways. *Blood* 1993; 82: 182-191.
- Watson ML, Kingsmore SF, Johnston GI, Sieglman MH, Le Beau MM, Lemons RS, Bara NS, Howard TA, Weissman IL, McEver RP, Seldin MF (1990). Genomic organisation of the Selectin Family of leucocyte adhesion molecules on human and mouse chromosome-1. *Journal of Experimental Medicine* 172: 263-272.
- Watt SM, Williamson J, Genevier H, Nesbitt SA, Hatzfeld A, Fawcett J, Simmons DL, Edwards AJ, van Dongen JJM, Coombe DR (1993). The heparin binding PECAM-1 adhesion molecule is expressed by CD34 positive hematopoietic precursor cells with early myeloid and B lymphoid cell phenotypes. *Blood* 82: 2649-2663.
- Weichel W, Irlenbusch S, Kato K, Radbruch A (1992). Sorting of rare cells. *In*: Radbruch A *ed.* Flow cytometry and cell sorting. Berlin: Springer-Verlag. 159-167.
- Whitlock CA, Witte ON (1982). Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proceedings of the National Academy of Sciences USA* 79: 3608-3612.
- Williams AF (1987). A year in the life of immunoglobulin superfamily. *Immunology Today* 8: 298-303.
- Williams AF, Barclay AN (1988). The immunoglobulin superfamily: domains for cell surface recognition. *Annual Reviews in Immunology* 6: 381-405.
- Williams AF (1991). Out of equilibrium. *Nature* 352: 473-474.
- Williams DA, Rios M, Stephens C, Patel VP (1991). Fibronectin and VLA-4 in haematopoietic stem cell - microenvironment interactions. *Nature* 352: 438-441.



- Williams DA (1993). Ex vivo expansion of hematopoietic stem and progenitor cells - robbing Peter to pay Paul? *Blood* 81: 3169-3172.
- Wilkins JA, Stupack D, Stewart S, Caixia S (1991).  $\beta 1$  integrin-mediated lymphocyte adherence to extracellular matrix is enhanced by phorbol ester treatment. *European Journal of Immunology* 21: 517-522.
- Winslow JM, Liesveld JL, Ryan DH, Dippersio JF, Abboud CN (1994). CD34+ progenitor cell isolation from blood and marrow: a comparison of techniques for small-scale selection. *Bone Marrow Transplantation* 14: 265-271.
- Winton EF, Colenda KW (1987). Use of long-term marrow cultures to demonstrate progenitor cell precursors in marrow treated with 4- hydroperoxycyclophosphamide. *Experimental Hematology* 15: 710-714.
- Wolpert L (1969). Positional information and the spatial pattern of cellular differentiation. *Journal of Theoretical Biology* 25: 1-47.
- Wu AM, Siminovitch L, Till JE, McCulloch EA (1968). Evidence for a relationship between mouse hemopoietic stem cells and cells forming colonies in culture. *Proceedings of the National Academy of Sciences USA* 59: 1209-1215.
- Wu DD, Keating A (1993). Hematopoietic stem cells engraft in untreated transplant recipients. *Experimental Hematology* 21: 251- 256.
- Wunder E, Sovalat H, Fritsch G, Silvestri F, Henon P, Serke S (1992). Report on the European workshop on peripheral blood stem cell determination and standardization, Mulhouse, France. February 6-8th and 14-15th 1992. *Journal of Hematotherapy* 1: 131-142.
- Wunder E, Bacrenzung M, Sovalat H, Henon Ph (1992b). Comparison of hematopoietic cells in steady state and during regeneration in blood and bone marrow. *Experimental Hematology* 20: 732 *abstract*.
- Wunder E, de Wynter E (1994). Basics of CD34<sup>+</sup> cell purification, evaluation and use of the separated products. *In*: Wunder E, Sovalat H, Hénon PR, Serke S *eds*. Hematopoietic Stem Cells. The Mulhouse Manual. AlphaMed Press, Ohio, USA. 125-131.
- Wunder E, Sovalat H, Hénon PR, Serke S *eds* (1994). Hematopoietic Stem Cells. The Mulhouse Manual. AlphaMed Press, Ohio, USA.
- Young PE, Baumhueter S, Lasky LA (1995). The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine development. *Blood* 85: 96-105.

Yurchenco PD, Schnitty JC (1990). Molecular architecture of basement membranes. *FASEB Journal* 4: 1577-1590.

Zanjani E, Pallavicini MG, Ascensao JL, Flake AW, Langlois RG, Reitsma M, MacKintosh FR, Stutes D, Harrison M, Tavassoli M (1992). Engraftment and long-term expression of human fetal hemopoietic stem cells in sheep following transplantation in utero. *Journal of Clinical Investigation* 89: 1178-1188.

Zauli G, Furlini G, Vitale M, Re MC, Gibellini D, Zamai L, Visani G, Borgatti P, Capitani S, La Placa M (1994). A subset of human CD34<sup>+</sup> hematopoietic progenitors express low levels of CD4, the high affinity receptor for human immunodeficiency virus-type I. *Blood* 84: 1896-1905.

Zuckerman KS, Wicha MS (1983). Extracellular matrix production by the adherent cells of long term murine bone marrow cultures. *Blood* 61: 540-547.

Zuckerman KS, Prince CW, Gay S (1989). The hemopoietic extracellular matrix. *In*: Tavassoli M *ed.* *Handbook of the Hemopoietic Microenvironment*. Humana Press, New Jersey: 399-432.

## **APPENDICES.**

### **Appendix 1. Equipment, Materials, Monoclonal Antibodies and Cell Lines.**

***p 357***

*1.1 Equipment. p 357.*

*1.2 Materials. p 357.*

*1.3 Monoclonal antibodies. p 359.*

*1.4 Cell lines. p 362.*

### **Appendix 2 Preparation of Solutions and Media.**

***p 363***

*2.1 Tissue Culture Solutions. p 363.*

*2.2 Immunocytometry Solutions. p 364.*

### **Appendix 3. Additional Tables.**

***p 365***

### **Appendix 4. Human Bone Marrow Endothelial Cultures.**

***p 385***

### **Appendix 5. Publications.**

***p 388***

## **Appendix 1 Equipment, Materials, Monoclonal Antibodies and Cell Lines.**

### *1.1 Equipment.*

#### *1.1.1 Specific Equipment.*

ZF. Coulter Electronics, Dunstable, UK.

T-890. Coulter Electronics, Dunstable, UK.

FACScan Flow Cytometer (FACS). Becton Dickinson, California, USA.

Dynal system. Gift of Dr Torstein Egeland, University of Oslo, Norway.

*miniMACS* system. Miltenyi Biotec, Cologne, Germany.

FACStar Plus Flow Sorter. Becton Dickinson, California, USA.

Gamma Counter. Auto-Gamma 500. Packard Instruments, Reading, UK.

Micro-Osmometer. Advanced Instruments Inc., Massachusetts, USA.

pH meter. Ciba Corning Diagnostic Ltd., Sudbury, UK.

12 & 24 well tissue culture grade plates. Costar UK Ltd., High Wycombe, UK.

25cm<sup>2</sup> and 75cm<sup>2</sup> canted neck tissue culture flasks. Greiner, Dursley, UK..

#### *1.1.2 Computer software.*

Consort 30. Becton Dickinson, California, USA.

Lysis II. Becton Dickinson, California, USA.

Paint-A-Gate. Becton Dickinson, California, USA.

### *1.2 Materials.*

A23187 Calcium ionophore. Sigma Chemical Co. Poole, UK.

Acetic acid. BDH Laboratory Supplies, Poole, UK.

Ammonium chloride. BDH Laboratory Supplies, Poole, UK.

Arginine-Glycine-Asparagine-Serine [RGDS] (synthetic peptide). Sigma Chemical Co. Poole, UK.

Arginine-Glycine-Glutamine-Serine [RGES] (synthetic peptide). Sigma Chemical Co. Poole, UK.

Bovine serum albumin (BSA). Sigma Chemical Co. Poole, UK.

Chondroitin Sulphate A (sodium salt from bovine trachea). Sigma Chemical Co. Poole, UK.

Chondroitinase ABC (from *Proteus vulgaris*). Sigma Chemical Co. Poole, UK.

Collagen Type I (acid soluble from human placenta). Sigma Chemical Co. Poole, UK.

Collagen Type III (acid soluble from human placenta). Sigma Chemical Co. Poole, UK.

Collagen Type IV (acid soluble from human placenta) Sigma Chemical Co. Poole, UK.

Dimethylsulfoxide (DMSO). Sigma Chemical Co. Poole, UK.

Deoxyribonuclease I. Sigma Chemical Co. Poole, UK.

Ethylenediaminetetra-acetic acid (EDTA). BDH Laboratory Supplies, Poole, UK.

Fibronectin (from human plasma). Sigma Chemical Co. Poole, UK.

Fibronectin (from human foreskin fibroblasts). Sigma Chemical Co. Poole, UK.

Ficoll-Hypaque (density 1.077g/dl) Lymphoprep. Nycomed Pharma, Oslo, Norway.

Foetal calf serum (FCS). Sigma Chemical Co. Poole, UK.

Gentamycin 5mg/ml. Sigma Chemical Co. Poole, UK.

Glutamax-1. Gibco BRL., Paisley, UK.

Hanks' Balanced Salts Solution (HBSS).without Ca or Mg, sodium bicarbonate added. Northumbria Biologicals. Cramlington UK.

Heparan Sulphate (sodium salt from bovine kidney). Sigma Chemical Co. Poole, UK.

Heparin (mucous) Inj BP. Leo Laboratories Ltd, Princes Risborough, UK.

Heparinase I (from *Flavobacterium heparinum*). Sigma Chemical Company, Poole, UK.

Horse Serum. Sigma Chemical Company, Poole, UK.

Human gamma globulin (Cohn fractions II and III). Sigma Chemical Company, Poole, UK.

Hyaluronic Acid (from human umbilical cord). Sigma Chemical Co. Poole, UK.

Hyaluronidase (from bovine testes). Sigma Chemical Company, Poole, UK.



Iscove's Modified Dulbecco's Medium (IMDM) with L Glutamine and 25mM HEPES. Sodium bicarbonate added. Gibco BRL. Paisley, UK.

Laminin (from human placenta). Sigma Chemical Co. Poole, UK.

Lyserglobin. JT Baker BV. Deventer, Holland.

Magnesium Sulphate. Sigma Chemical Co. Poole, UK.

Methylcellulose. Sigma Chemical Co. Poole, UK.

Mouse Serum. Prepared by Kay Samuel.

Non-Idet P40 (non-ionic detergent). Sigma Chemical Co. Poole, UK.

Paraformaldehyde. BDH Laboratory Supplies, Poole, UK.

Penicillin 10,000u/ml, Streptomycin 10mg/ml. Sigma Chemical Co. Poole, UK.

Phosphate buffered saline (PBS). Sigma Chemical Company, Poole, UK.

Quantum simply cellular beads. Sigma Chemical Co. Poole, UK.

Rabbit Serum. Scottish Antibody Production Unit. Carlisle, UK.

RPMI 1640. Scottish National Reagents Unit.

Sodium azide. Sigma Chemical Co. Poole, UK.

Sodium chromate ( $^{51}\text{Cr}$ ). Amersham International PLC. Amersham, UK.

Sodium hydrogen carbonate. BDH Laboratory Supplies, Poole, UK.

Thrombospondin (from human platelets). Sigma Chemical Co. Poole, UK.

Trypan blue stain (0.4%). Sigma Chemical Co. Poole, UK.

Trypsin/EDTA (0.25%). Sigma Chemical Co. Poole, UK.

Vitronectin (from human placenta). Sigma Chemical Co. Poole, UK.

Water (distilled), prepared by distillation within the laboratory.

### *1.3 Monoclonal antibodies.*

#### *1.3.1 CD classified.*

##### *CD7:*

8H8.1 (FITC), IgG2a (murine). Immunotech, Marseille, France.

##### *CD19:*

J4.119 (FITC), IgG1 (murine). Immunotech, Marseille, France.

*CD11a (LFA-1 $\alpha$  chain):*

IOT16 (Purified), IgG1 (murine). Immunotech, Marseille, France.

*CD31 (PECAM-1, GpIIa):*

5.6E (Purified), IgG1 (murine). Immunotech, Marseille, France.

L133.1 (Purified), IgG1 (murine). Becton Dickinson, Oxford, UK.

*CD33:*

D3HL60 (FITC), IgG1 (mouse). Immunotech, Marseille, France.

*CD34:*

My10 [HPCA-1] (Purified), IgG1 (murine). Becton Dickinson, Oxford, UK.

8G12 [HPCA-2] (RPE), IgG1 (murine) Becton Dickinson, Oxford, UK.

QBEND10 (Purified), IgG1 (murine). Immunotech, Marseille, France.

QBEND-10 (FITC), IgG1 (murine). Immunotech, Marseille, France.

BI-3C5 (Purified), IgG1 (murine) Sera-Lab, Sussex, UK.

Tük-3 (Purified), IgG3 (murine) Dako, High Wycombe, UK.

12.8 (Purified), IgM (murine) a gift of Dr ID Bernstein, Seattle.

*CD36:*

FA6-152 (Purified), IgG1 (murine). Immunotech, Marseille, France.

*CD38:*

T16 (FITC), IgG1 (murine). Immunotech, Marseille, France.

*CD41a (Gp IIb):*

P2 (FITC), IgG1 (murine). Immunotech, Marseille, France.

*CD44 (HCAM):*

F10-44-2 (Purified), IgG2a (murine). Serotec, Oxford, UK.

L178 (Purified), IgG1 (murine). Becton Dickinson, Oxford, UK.

*CD45 (Leucocyte common antigen):*

IOL1a (Purified), IgG1 (murine). Immunotech, Marseille, France.

IOL1c (FITC), IgG1 (murine). Immunotech, Marseille, France.

*CD49d (VLA-4 $\alpha$ -chain,  $\alpha_4$ ):*

HP2/1 (Purified), IgG1 (murine). Immunotech, Marseille, France.

L25.3 (Purified), IgG2b (murine). Becton Dickinson, Oxford.

*CD49e (VLA-5  $\alpha$ -chain,  $\alpha_5$ ):*

SAM1 (Purified), IgG2a (murine). Immunotech, Marseille, France.

mAb 16 (purified), IgG2a (murine). Becton Dickinson, Oxford, UK.

*CD51 (VNR  $\alpha$ -chain):*

AMF7 (Purified), IgG1 (murine). Immunotech, Marseille, France.

*CD54 (ICAM-1):*

84HIO (Purified), IgG1 (murine). Immunotech, Marseille, France.

LB-2 (purified), IgG2b (murine). Becton Dickinson, Oxford, UK.

*CD58 (LFA-3):*

AMF7 (Purified), IgG2a (murine). Immunotech, Marseille, France.

*CD62L (L-Selectin):*

Dreg56 (Purified), IgG1 (murine). Immunotech, Marseille, France.

*CD71 (transferrin receptor):*

YDJ1.2.2 (FITC), IgG1 (murine). Immunotech, Marseille, France..

### *1.3.2 Unclassified.*

*HLA-DR:*

B8.12.2 (FITC). IgG2b (murine). Immunotech, Marseille, France.

### *1.3.3 Irrelevant specificity.*

IgG1 (Purified), (murine), Immunotech, Marseille, France.

IgG2a (Purified), (murine), Immunotech, Marseille, France.

IgG1 (FITC), (murine), Immunotech, Marseille, France.

IgG1 (RPE), (murine), Immunotech, Marseille, France.

### *1.3.4 Second and third-step reagents.*

Sheep F(ab')<sub>2</sub> anti-mouse IgG -FITC conjugate (adsorbed with human serum).

Sigma Chemical Co. Poole, UK.

Sheep F(ab')<sub>2</sub> anti-mouse IgG -RPE conjugate (adsorbed with human serum).

Sigma Chemical Co. Poole, UK

Goat F(ab')<sub>2</sub> anti-mouse IgG - Red 613. Gibco BRL. Paisley, UK.

Goat anti-mouse IgG (Fab specific) -biotin conjugate. Sigma Chemical Co. Poole, UK.

Streptavidin-Quantum Red conjugate. Sigma Chemical Co. Poole, UK.

Rat anti-mouse IgG2a -PerCP conjugate. (Peridinin Chlorophyll Protein). Becton Dickinson, Oxford, UK.

Rat anti-mouse IgG1 -microbead conjugate. Miltenyi Biotec Inc, Cologne, Germany.

#### *1.4 Cell lines.*

M210-B4 murine stromal cell line. A gift of Dr Connie Eaves, Terry Fox Laboratory, University of British Columbia, Vancouver.

5637 human bladder carcinoma cell line. American Tissue Culture Collection, Rockville MD, USA.

KG1a human acute myelogenous leukaemia cell line. American Tissue Culture Collection, Rockville MD, USA.

K562. human chronic myelogenous leukaemia. A gift of Dr David Flavell, Department of Pathology, University of Southampton.

HL60. human promyelocytic leukaemia. A gift of Dr David Flavell, Department of Pathology, University of Southampton.

CEM human acute T lymphoblastic leukaemia. A gift of Dr David Flavell, Department of Pathology, University of Southampton.

NALM-6 human B cell lymphoma line. A gift of Dr David Flavell, Department of Pathology, University of Southampton.

## **Appendix 2 Preparation of Solutions and Media.**

### *2.1 Tissue Culture Solutions.*

#### *Antibiotic stock (20ml):*

penicillin 10,000u/ml, streptomycin 10mg/ml	7.0ml
gentamycin 5mg/ml	1.4ml
distilled water	11.6ml

#### *Standard tissue culture medium (500ml):*

Iscove's modified Dulbecco's medium (IMDM)	440ml.
foetal calf serum (FCS).	50ml
glutamax-1	5ml
antibiotic stock	5ml

#### *CFU-GM assay medium (Stock 1.8ml):*

9% methylcellulose	800µl
foetal calf serum (FCS)	600µl
5637 conditioned medium	200µl
Iscove's modified Dulbecco's medium (IMDM)	170µl
antibiotic stock	30µl

#### *LTBMC medium (500ml):*

Iscove's modified Dulbecco's medium [IMDM]	400ml
foetal calf serum [FCS]	50ml
horse serum	50ml
antibiotic stock	5ml
hydrocortisone ( $10^{-4}$ M)	5ml



## *2.2 Immunocytochemistry Solutions.*

Immunocytochemistry handling medium (100ml).

phosphate buffered saline (PBS)	100ml
bovine serum albumin (BSA)	1g
sodium azide	0.1g
ethylenediaminetetra-acetic acid (EDTA)	0.02g

*Human gamma-globulin solution (100ml).*

phosphate buffered saline (PBS)	100ml
human gamma globulin (Cohn fraction II and III)	0.5g

*Red cell lysis solution (100ml).*

dH <sub>2</sub> O	100ml
ammonium chloride	830mg
ethylenediaminetetra-acetic acid (EDTA)	4mg
sodium hydrogen carbonate	84mg

### Appendix 3. Additional Tables.

**Table A1 Overall Accuracy of CD34<sup>+</sup> Quantitation.**

<i>Sample</i>	<i>mean <math>\pm</math> standard deviation</i>	<i>coefficient of variation</i>
PB	1.08 $\pm$ 0.16%	cv=14.8%
BM	1.36 $\pm$ 0.07%	cv=5.3%
LP	1.51 $\pm$ 0.09%	cv=6.0%
LP	0.18 $\pm$ 0.02%	cv=11.5%
LP	0.17 $\pm$ 0.02%	cv=12.1%
LP	0.41 $\pm$ 0.10%	cv=23.8%
CB	1.40 $\pm$ 0.27%	cv=19.3%
BM	0.13 $\pm$ 0.05%	cv=33.4%
PB	6.70 $\pm$ 0.22%	cv=3.2%
PB	2.78 $\pm$ 0.20%	cv=1.4%
LP	1.73 $\pm$ 0.55%	cv=31.7%
CB	0.76 $\pm$ 0.08%	cv=11.1%
CB	0.28 $\pm$ 0.12%	cv=43.5%
CB	0.34 $\pm$ 0.06%	cv=17.6%
LP	3.96 $\pm$ 0.72%	cv=18.1%

*Legend.* Refer to Figure 2.9. *PB:* Peripheral Blood. *LP:* Leucapheresis Product.

*BM:* Bone Marrow. *CB:* Cord Blood.

**Table A2 Correlation between CFU-GM and CD34<sup>+</sup> Cells in 14 Leucapheresis Product Samples.** (*Legend. Refer to Figure 2.10).*

<i>sample</i>	<i>CD34%<sup>+</sup></i>	<i>CFU-GM/10<sup>5</sup></i>	<i>Cloning Efficiency</i>
#1	0.1%	3	3.5%
#2	0.2%	1	0.75%
#3	0.0%	1	
#4	0.5%	2	0.4%
#5	0.2%	1	0.5%
#6	0.8%	17	2.1%
#7	1.9%	175	9.2%
#8	0.4%	7	1.7%
#9	0.1%	6	6.0%
#10	0.3%	10	3.3%
#11	0.0%	2	
#12	0.0%	3	
#13	3.0%	613	20.0%
#14	2.0%	650	32.5%

**Table A3 Analysis of CD34<sup>+</sup> Counts and CFU-GM in Normal Individuals.**

<i>samples</i>	<i>CD34+</i>	<i>CFU-GM/10<sup>5</sup></i>
#1	0.1%	0
#2	0.2%	1
#3	0.0%	3
#4	0.4%	3
#5	0.0%	0
#6	0.2%	1
#7	0.3%	1
#8	0.1%	0
#9	0.0%	1
#10	0.0%	0

*Legend.* Refer to pages 102.

**Table A4 Percentage Positivity Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood.**

<i>CD</i>	<i>BM</i>	<i>PB<sup>m</sup>#1</i>	<i>PB<sup>m</sup>#2</i>	<i>CB</i>
CD34 <sup>+</sup> %	5.06 ± 4.71 (8)	6.47 ± 3.91 (7)	6.08 ± 4.90 (8)	2.48 ± 1.92 (8)
CD34 <sup>n</sup>	510 ± 470	650 ± 390	610 ± 490	250 ± 190
CD54%	91 ± 11 p<0.001 (7)	83 ± 26 p<0.001 (7)	93 ± 11 p<0.001 (8)	56 ± 26% p<0.001 (8)
CD31%	91 ± 7 p<0.001 (7)	84 ± 27 p<0.01 (5)	94 ± 6% p<0.001 (8)	
CD58%	65 ± 25 p<0.001 (7)	32 ± 20 p<0.05 (6)	27 ± 18% p<0.01 (8)	19 ± 23% ns (7)
CD49d%	67 ± 25 p<0.001 (7)	59 ± 35 p<0.01 (7)	71 ± 23% p<0.001 (8)	44 ± 16% p<0.01 (6)
CD49e%	62 ± 19 p<0.001 (8)	34 ± 34 ns (7)	32 ± 19% p<0.01 (8)	28 ± 20% ns (5)
CD11a%	84 ± 12 p<0.001 (8)	84 ± 13 p<0.001 (7)	78 ± 15% p<0.001 (8)	62 ± 20% p<0.001 (8)
CD51%	9 ± 7 ns (7)	9 ± 12 ns (7)	5 ± 6% ns (8)	11 ± 6% ns (6)

*Legend.* Refer to Figure 3.2 and overpage.



**Table A4 continued.**

<i>CD</i>	<i>BM</i>	<i>PB<sup>m</sup>#1</i>	<i>PB<sup>m</sup>#2</i>	<i>CB</i>
CD62L%	64 ± 22 p<0.001 (7)	58 ± 34 p<0.01 (7)	60 ± 28% p<0.001 (7)	19 ± 8 p<0.001 (6)
CD44%	98 ± 2 p<0.001 (8)	99 ± 2 p<0.001 (7)	97 ± 5% p<0.001 (8)	93 ± 5% p<0.001(7)
CD36%	20 ± 11 p<0.01 (8)	29 ± 14 p<0.01 (7)	14 ± 14% ns (8)	19 ± 9% p<0.01 (7)
<i>FITC controls</i>				
IgG1	0.48 ± 0.05% (8)	0.51 ± 0.09% (7)	0.50 ± 0.00% (8)	0.51 ± 0.04% (8)
CD45	71 ± 12% p<0.001 (6)	83 ± 5% p<0.001 (5)	88 ± 9% p<0.001 (8)	92 ± 6% p<0.001 (4)
<i>RPE controls</i>				
IgG1	4.9 ± 0.1% (8)	4.5 ± 0.4% (7)	5.0 ± 0.3% (8)	4.9 ± 0.2% (8)
IgG2a	4.9 ± 0.2% (8)	4.9 ± 0.3% (7)	5.0 ± 0.4% (8)	5.0 ± 0.1% (8)
CD45	69 ± 12% p<0.001 (6)	80 ± 9% p<0.01 (3)	87 ± 9% p<0.001 (5)	88 ± 10% p<0.001 (4)

*Legend.* Refer to Figure 3.2. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired *t* test.

**Table A5 Mean Fluorescence Intensity of Cell Adhesion Molecule Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood.**

<i>CD</i>	<i>BM</i>	<i>PB<sup>m</sup>#1</i>	<i>PB<sup>m</sup>#2</i>	<i>CB</i>
CD54	69 ± 28 p<0.01	84 ± 81 ns	68 ± 52 ns	128 ± 82 p<0.05
CD31	66 ± 31 p<0.01	69 ± 43 p<0.05	44 ± 55 ns	
CD58	30 ± 7 p<0.01	50 ± 36 p<0.05	57 ± 92 ns	68 ± 44 ns
CD49d	40 ± 17 p<0.01	53 ± 58 ns	41 ± 27 ns	65 ± 27 p<0.05
CD49e	33 ± 12 p<0.001	40 ± 21 p<0.05	31 ± 19 ns	52 ± 25 ns
CD11a	72 ± 39 p<0.01	66 ± 36 p<0.01	60 ± 65 ns	109 ± 63 p<0.05
CD51	14 ± 4 ns	25 ± 31 ns	11 ± 7 ns	26 ± 6 ns
CD62L	44 ± 23 p<0.01	45 ± 35 p<0.05	40 ± 34 ns	44 ± 23 ns
CD44	329 ± 232 p<0.01	359 ± 233 p<0.05	444 ± 434 ns	454 ± 263 p<0.02
CD36	48 ± 25 p<0.01	49 ± 30 p<0.05	33 ± 26 ns	66 ± 10 p<0.01

*Legend.* Refer to Figure 3.3 and overpage.

**Table A5 continued.**

<i>CD</i>	<i>BM</i>	<i>PB<sup>m</sup>#1</i>	<i>PB<sup>m</sup>#2</i>	<i>CB</i>
<i>FITC controls</i>				
IgG1	12 ± 3	13 ± 9	10 ± 6	14 ± 8
<i>RPE controls</i>				
IgG1	9 ± 3	10 ± 4	9 ± 3	23 ± 15
IgG2a	10 ± 3	12 ± 3	12 ± 5	32 ± 19

*Legend.* Refer to Figure 3.3. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired *t* test.

**Table A6 Percentage Positive Lineage Marker Expression within the CD34<sup>+</sup> Populations of Bone Marrow, Mobilised Peripheral Blood and Cord Blood.**

<i>CD</i>	<i>BM (n=5)</i>	<i>PB<sup>m</sup> (n=4)</i>	<i>LP (n=4)</i>	<i>CB (n=4)</i>
CD34%	4.13 ± 1.69	3.10 ± 3.40	4.81 ± 1.56	0.57 ± 0.40
CD34n	1,232 ± 512	553 ± 264	1,423 ± 429	86 ± 73
IgG1	0.06 ± 0.01	0.03 ± 0.02	0.07 ± 0.05	0.05 ± 0.01
Contam (%)	1.5 ± 0.6%	0.8 ± 0.6%	1.5 ± 1.0%	12.2 ± 6.5%
CD33%	11 ± 11 ns	6 ± 4 ns	2 ± 2 ns	7 ± 7 ns
CD7%	8 ± 8 ns	5 ± 2 ns	2 ± 1 ns	14 ± 7 p<0.05
CD19%	16 ± 21 ns	4 ± 2 ns	1 ± 1 ns	7 ± 6 ns
CD71%	40 ± 27 p<0.05	33 ± 21 ns	24 ± 9 ns	44 ± 20 p<0.05
CD41a%	8 ± 6 p<0.05	5 ± 3 ns	7 ± 5 ns	10 ± 3 ns
CD38%	68 ± 31 p<0.01	63 ± 21 p<0.01	63 ± 13 p<0.01	22 ± 5 p<0.01
HLA-DR%	76 ± 17 p<0.001	85 ± 6 p<0.001	88 ± 8 p<0.001	77 ± 18 p<0.01
IgG1%	2 ± 3	2 ± 1	2 ± 2	4 ± 6
CD45%	76 ± 35 p<0.01	97 ± 5 p<0.001	99 ± 1 p<0.01	96 ± 3 p<0.001

*Legend.* Refer to Figure 3.6. Results are quoted as the mean ± standard deviation of (n) experiments. Comparisons are made to the negative controls by paired *t* test.

**Table A7 Mean Fluorescence Intensity of Lineage Marker Expression within CD34<sup>+</sup> Populations from Bone Marrow, Mobilised Peripheral Blood and Umbilical Cord Blood.**

<i>CD</i>	<i>BM</i>	<i>PB<sup>m</sup></i>	<i>LP</i>	<i>CB</i>
CD33	30 ± 34 ns	12 ± 7 ns	3 ± 3 ns	7 ± 4 ns
CD7	29 ± 31 ns	11 ± 8 ns	3 ± 1 ns	4 ± 4 ns
CD19	29 ± 31 p<0.05	8 ± 5 ns	3 ± 1 ns	9 ± 3 ns
CD71	53 ± 29 p<0.01	23 ± 9 ns	16 ± 7 p<0.05	495 ± 502 ns
CD41a	34 ± 32 ns	12 ± 7 ns	17 ± 19 ns	43 ± 60 ns
CD38	83 ± 14 p<0.001	35 ± 5 p<0.01	24 ± 6 p<0.01	14 ± 6 ns
HLA-DR	166 ± 26 p<0.01	85 ± 43 p<0.05	50 ± 18 ns	96 ± 32 p<0.05
<i>FITC controls</i>				
IgG1	21 ± 24	8 ± 3	4 ± 1	8 ± 6
CD45%	96 ± 22 p<0.001	126 ± 30 p<0.01	125 ± 27 p<0.05	78 ± 4 p<0.01

*Legend:* Refer to Figure 3.7. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired *t* test.



**Table A8 Cell Adhesion Molecules Expressed by KG1a.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments.</i>
CD54	99 ± 3% p<0.001	56 ± 18 p<0.05	n=3
CD31	30 ± 13% p<0.05	18 ± 7 p<0.01	n=3
CD58	98 ± 1% p<0.001	44 ± 13 p<0.05	n=3
CD49d	99 ± 1% p<0.001	58 ± 11 p<0.05	n=3
CD49e	99 ± 1% p<0.001	52 ± 16 p<0.05	n=3
CD11a	99 ± 0% p<0.001	168 ± 61 p<0.05	n=3
CD51	7 ± 3% ns	5 ± 0	n=3
CD62L	5 ± 4% ns	5 ± 1 ns	n=3
CD44	99 ± 0% p<0.001	307 ± 24 p<0.01	n=3
CD36	56 ± 39% ns	157 ± 252 ns	n=3
<i>Controls.</i>			
IgG1 SAM-FITC	2 ± 1%	4 ± 0	n=3
IgG2a SAM-FITC	2 ± 3%	4 ± 1	n=3
CD45 SAM-FITC	99 ± 1% p<0.01	113 ± 5 p<0.01	n=2

**Table A8 continued. Lineage and Activation Markers Expressed by KG1a.**

<i>Antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD34	70 ± 19%		n=4
CD33	11 ± 8% ns	8 ± 6 ns	n=4
CD7	48 ± 27% p<0.05	42 ± 15 p<0.01	n=4
CD19	4 ± 1% ns	4 ± 2 ns	n=4
CD71	84 ± 8% p<0.001	128 ± 25 p<0.05	n=4
CD41a	3 ± 1% ns	3 ± 1 ns	n=4
CD38	9 ± 4% p<0.05	7 ± 4 ns	n=4
HLA-DR	3 ± 1% ns	3 ± 1 ns	n=4
<i>Controls.</i>			
IgG1-FITC	3 ± 1%	3 ± 1	n=4
CD45-FITC	93 ± 4% p<0.001	113 ± 46 p<0.01	n=4

*Legend.* Refer to Figures 3.9-3.12. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired *t* test.

**Table A.9 Cell Adhesion Molecule Expression by K562.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD54	66 ± 10% p<0.01	50 ± 6 p<0.001	n=4
CD31	24 ± 26% ns	22 ± 12 ns	n=4
CD58	60 ± 8% p<0.01	37 ± 9 p<0.01	n=4
CD49d	44 ± 8% p<0.01	31 ± 7 p<0.01	n=4
CD49e	74 ± 6% p<0.001	55 ± 16 p<0.05	n=4
CD11a	19 ± 14% ns	21 ± 2 ns	n=4
CD51	58 ± 13% p<0.01	47 ± 8 p<0.01	n=4
CD62L	11 ± 17% ns	11 ± 4 ns	n=4
CD44	21 ± 3% ns	25 ± 6 ns	n=3
CD36	17 ± 3% ns	23 ± 3 ns	n=4
<i>Controls.</i>			
IgG1 SAM-FITC	16 ± 4	19 ± 5	n=4

**Table A.9 continued: Lineage and Activation Markers Expressed by K562.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD34	3 ± 1% ns	9 ± 4 ns	n=3
CD33	12 ± 8% ns	12 ± 7 ns	n=4
CD7	13 ± 18% ns	14 ± 3 ns	n=4
CD19	19 ± 4% p<0.01	19 ± 4 p<0.05	n=4
CD71	65 ± 16% p<0.05	38 ± 21 ns	n=4
CD41a	10 ± 3% ns	11 ± 4 ns	n=4
CD38+	10 ± 4% ns	11 ± 4 ns	n=4
HLA-DR	12 ± 2% ns	12 ± 7 ns	n=4
<i>Controls.</i>			
IgG1-FITC	10 ± 4%	10 ± 4	n=4
CD45-FITC	90 ± 5% p<0.001	69 ± 35 p<0.05	n=4

*Legend.* Refer to Figures 3.9-3.12. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired *t* test.

**Table A.10 Cell Adhesion Molecule Expression by HL60.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD54	81 ± 14% p<0.01	72 ± 35 p<0.05	n=4
CD31	47 ± 27% ns	61 ± 36 ns	n=4
CD58	68 ± 16% p<0.01	57 ± 54 ns	n=4
CD49d	82 ± 22% p<0.01	83 ± 40 p<0.05	n=4
CD49e	73 ± 26% p<0.01	61 ± 28 p<0.05	n=4
CD11a	79 ± 25% p<0.01	67 ± 19 p<0.01	n=4
CD51	30 ± 45 ns	26 ± 1 ns	n=4
CD62L	3 ± 2% ns	19 ± 9 p<0.05	n=4
CD44	74 ± 1%	311 ± 390	n=2
CD36	18 ± 17% ns	28 ± 13 ns	n=4
<i>Controls.</i>			
IgG1 SAM-FITC	5 ± 5%	19 ± 8	n=4
IgG2a SAM-FITC	5 ± 5%	4 ± 0.1	n=4
CD45 SAM-FITC	87 ± 9% p<0.001	103 ± 46 p<0.05	n=4



**Table A.10 continued: Lineage and Activation Marker Expression by HL60.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD34	5 ± 2% ns	9 ± 4 ns	n=4
CD33	27 ± 27% ns	14 ± 9 ns	n=4
CD7	32 ± 20% ns	15 ± 3 ns	n=4
CD19	22 ± 9% p<0.05	14 ± 5 ns	n=4
CD71	30 ± 16% ns	21 ± 21 ns	n=4
CD41a	9 ± 7% ns	10 ± 8 ns	n=4
CD38	26 ± 27% ns	15 ± 13 ns	n=4
HLA-DR	31 ± 18% p<0.05	15 ± 6 p<0.05	n=4
<i>Controls.</i>			
IgG1-FITC	5 ± 2%	9 ± 4	n=4
CD45-FITC	95 ± 3% p<0.001	105 ± 82 ns	n=4

*Legend.* Refer to Figures 3.9-3.12. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired *t* test.

**Table A.11 Cell Adhesion Molecule Expression by NALM-6.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD54	82 ± 5% p<0.001	40 ± 7 p<0.01	n=4
CD31	1 ± 0.5% ns	4 ± 0.4 ns	n=2
CD58	84 ± 9% p<0.001	36 ± 3 p<0.001	n=4
CD49d	79 ± 14% p<0.01	35 ± 9 p<0.01	n=4
CD49e	68 ± 25% p<0.02	43 ± 21 p<0.05	n=4
CD11a	2 ± 2.5% ns	7 ± 4 ns	n=4
CD51	4 ± 4% ns	9 ± 4 ns	n=4
CD62L	0.4 ± 0.2% ns	4 ± 1 ns	n=4
CD44	0.7 ± 0.3% ns	5 ± 4 ns	n=4
CD36	1 ± 0.6% ns	4 ± 2 ns	n=4
<i>Controls.</i>			
IgG1 SAM-FITC	0.8 ± 0.6%	4 ± 1	n=4
IgG2a SAM-FITC	0.9 ± 0.9%	5 ± 1	n=4
CD45 SAM-FITC	0.7 ± 0.5% ns	4 ± 1 ns	n=4

**Table A.11 continued: Lineage and Activation Marker expression by NALM-6.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD34	0.6 ± 0.4% ns	2 ± 1 ns	n=4
CD33	0.4 ± 0.2% ns	2 ± 0.3 ns	n=4
CD7	3 ± 2% ns	3 ± 2 ns	n=4
CD19	80 ± 22% p<0.01	21 ± 5 p<0.01	n=4
CD71	83 ± 17% p<0.01	34 ± 10 p<0.01	n=4
CD41a	2 ± 1% ns	2 ± 0.3 ns	n=4
CD38	85 ± 13% p<0.001	23 ± 2 p<0.001	n=4
HLA-DR	96 ± 4% p<0.001	64 ± 19 p<0.01	n=4
<i>Controls.</i>			
IgG1-FITC	2 ± 2%	3 ± 0.5	n=4
CD45-FITC	3 ± 2% ns	3 ± 0.4 ns	n=4

*Legend.* Refer to Figures 3.9-3.12. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired t test.

**Table A.12 Cell Adhesion Molecule Expression by CEM.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD54	56 ± 28% p<0.05	41 ± 24 p<0.05	n=4
CD31	19 ± 22%	27 ± 12	n=2
CD58	30 ± 29% ns	20 ± 3 p<0.02	n=4
CD49d	63 ± 31% p<0.05	44 ± 19 p<0.02	n=4
CD49e	23 ± 31% ns	20 ± 6 p<0.02	n=4
CD11a	61 ± 39% ns	40 ± 8 p<0.01	n=3
CD51	6 ± 6% ns	14 ± 16 ns	n=4
CD62L	6 ± 8% ns	9 ± 4 ns	n=4
CD44	84 ± 15% p<0.01	129 ± 35 p<0.01	n=4
CD36	2 ± 1% ns	8 ± 9 ns	n=4
<i>Controls.</i>			
IgG1 SAM-FITC	0.75 ± 0.5	7 ± 8	n=4
IgG2a SAM-FITC	1.2 ± 0.8	8 ± 7	n=4
CD45 SAM-FITC	87 ± 17 p<0.01	94 ± 15 p<0.01	n=4

**Table A.12 continued: Lineage and Activation Marker Expression by CEM.**

<i>antigen</i>	<i>% positivity</i>	<i>mean fluorescence intensity</i>	<i>number of experiments</i>
CD34	1 ± 2% ns	3 ± 1 ns	n=3
CD33	0.3 ± 0.3% ns	2 ± 1 ns	n=3
CD7	90 ± 6% p<0.001	52 ± 5 p<0.001	n=4
CD19	5 ± 2% ns	5 ± 2 ns	n=4
CD71	92 ± 5% p<0.001	86 ± 11 p<0.001	n=4
CD41a	1 ± 1% ns	3 ± 1 ns	n=4
CD38	74 ± 28% p<0.02	31 ± 11 p<0.02	n=4
HLA-DR	2 ± 1% ns	4 ± 1 ns	n=4
<i>Controls.</i>			
IgG1-FITC	2 ± 2%	3 ± 1	n=4
CD45	91 ± 12% p<0.001	69 ± 23 p<0.02	n=4

*Legend.* Refer to Figures 3.9-3.12. Results are quoted as the mean ± standard deviation of (n) replicate experiments. Comparisons are made to the appropriate negative controls by 2 tailed paired t test.

**Table A.13 Calibration using Microbeads.**

<i>bead population</i>	<i>mAb binding capacity</i>	<i>peak channel number</i>
Blank	0	0
Bead #1	4 095	10
Bead #2	15 493	31
Bead #3	35 340	60
Bead #4	134 309	220

Legend. Results are quoted as mean  $\pm$  standard error (number of replicate experiments). %<sup>+</sup> fluorescence and mfi is compared to isotype and fluorochrome-specific control by two-tailed *t* test. Refer to Figure 3.15.



#### **Appendix 4 Human Bone Marrow Endothelial Cultures.**

Human bone marrow endothelial cell cultures were prepared by John Sweetenham and Lisa Masek of the CRC Wessex Medical Oncology Unit, University of Southampton, as part of the collaborative work undertaken under a grant from the Leukaemia Research Fund.

Briefly, the method was as follows: a mononuclear cell preparation derived from normal human bone marrow was subject to positive selection using magnetic beads coated with the plant lectin *Ulex europeaus* agglutinin-1 (UEA-1). The cells were grown on tissue culture plates coated with ECM derived from human umbilical vein endothelial cells. Positive staining with several endothelial markers was observed including FVIII related antigen, BMA 120, CD31, ELAM-1, ICAM-1 (CD54) and E-Selectin (CD62E). Electron microscopy confirmed the presence of Weibel-Palade bodies. The cells have been cultured through up to 8 passages with no apparent change in their growth characteristics [Masek & Sweetenham, 1993; Masek, Sweetenham, Whitehouse *et al.* 1994]. A similar approach has recently been described by Rafii, Shapiro, Rimarachin *et al.* [1994].

A series of experiments was carried out to study the comparative adhesion of human haematopoietic cell lines to human umbilical vein endothelial cell cultures and endothelial LTBM using the  $^{51}\text{Cr}$  adhesion assay. 10ng/ml of recombinant human tumour necrosis factor  $\alpha$  was used to study the effects of an activating agent on the endothelial cultures. The use of the same methodological protocol and a series of parallel experiments on adhesion of  $^{51}\text{Cr}$ -labelled KG1a to pFn and dBSA, were sufficient to establish that the assay results were comparable between the Edinburgh and Southampton centres. The results of these experiments are presented in Table A.14. All five cell lines adhered significantly to endothelial cultures *in vitro*. Adherence of KG1a, HL60 and CEM was significantly greater to activated than to non-activated HUVECS ( $P < 0.001$ ), whilst K562 and NALM-6 demonstrated no difference. Adherence of KG1a, HL60 and CEM was significantly greater to non-activated endothelial LTBM than to non-activated HUVECS ( $P < 0.001$ ), whilst that of K562 and NALM-6 was similar. Adherence of all cell lines to

activated HUVECS was similar to that to activated endothelial LTBM. There was no difference in the adherence of any of the cell lines between activated and non-activated endothelial LTBM.

In summary of this data, it appears that some cell lines (KG1a, HL60, CEM) but not others (K562, NALM-6) are capable of responding to activation of HUVECS by tumour necrosis factor  $\alpha$ . Endothelial LTBM appear to be constitutively activated. These differences may be helpful in clarifying changes in ligand expression of functional importance during endothelial activation. This study demonstrates the reproducibility of the  $^{51}\text{Cr}$  adhesion assay between centres and the applicability of the method to examine the effects of manipulation of the substrate on HPC-stromal and -endothelial adhesion. In particular it may allow us to examine the proposition that chemotherapy, radiotherapy and cytokines give rise to HPC mobilisation through a direct effect on the bone marrow stroma or endothelium [Greenberger, 1991; Uhlman, Verfaillie, Jones *et al.* 1991; Shirota & Tavassoli, 1991 & 1992; Srour, 1994].

**Table A.14 Adhesion of Human Haematopoietic Cell Lines to Human Umbilical Vein and Bone Marrow Endothelial Cultures.**

<i>Substrate</i>	<i>KG1a</i>	<i>K562</i>	<i>HL60</i>	<i>CEM</i>	<i>NALM-6</i>
HUVECS	35.3 ± 4.7% p<0.001	46.0 ± 4.2% p<0.001	18.6 ± 7.4% p<0.001	22.5 ± 7.7% p<0.001	78.1 ± 7.0% p<0.001
HUVECS (activated)	85.0 ± 4.3% p<0.001	50.8 ± 4.3% p<0.001	68.5 ± 15% p<0.001	76.5 ± 2.3% p<0.001	87.4 ± 2.6% p<0.001
endothelial LTBMC	69.1 ± 4.3% p<0.001	50.2 ± 8.2% p<0.001	67.8 ± 7.9% p<0.001	47.4 ± 7.5% p<0.001	77.4 ± 4.9% p<0.001
endothelial LTBMC (activated)	77.1 ± 4.1% p<0.001	53.1 ± 5.8% p<0.001	76.7 ± 85% p<0.001	62.0 ± 9.8% p<0.001	81.4 ± 4.8% p<0.001
dBSA	2.9 ± 1.1	1.0 ± 0.3%	3.0 ± 0.8%	2.8 ± 1.5%	7.2 ± 2.8%

*Legend.* Results are quoted as the mean ± standard deviation of 4 replicate experiments. Comparison to dBSA by paired *t* test.

## **Appendix 5 Publications.**

Craig JIO, Turner ML, Parker AC. Peripheral blood stem cell transplantation. *Blood Reviews* 1992; 6: 59-67.

Turner ML. Cell adhesion molecules: a unifying approach to topographic biology. *Biological Reviews of the Cambridge Philosophical Society* 1992; 67: 359-377.

Turner ML. Human haematopoietic progenitors as a target for gene therapy. *Scottish Medical Journal* 1993; 38: 131-133.

Turner ML. Regulation of haematopoietic progenitor cell migration, mobilisation and homing. *Stem Cells* 1994; 12: 227-229.

Turner ML, McIlwiane K, Anthony RS, Parker AC. Differential expression of cell adhesion molecules by haematopoietic progenitors from bone marrow and adult peripheral blood. *Stem Cells* 1995; 13: 311-316.

# Peripheral Blood Stem Cell Transplantation

J. I. O. Craig, M. L. Turner, A. C. Parker

**SUMMARY.** Haematopoietic stem cells are usually sessile within the bone marrow microenvironment. However, small numbers do circulate in the peripheral blood of normal individuals, and following chemotherapy and/or intravenous growth factors, a substantial transient rise in circulating stem cells occurs. Leukocytes harvested by cytapheresis at this time can be used for autologous reconstitution of the haematopoietic and lymphoid systems following high dosage chemo/radiotherapy for the treatment of malignant disease. Peripheral blood stem cell transplants give rise to similar disease response rates as autologous bone marrow transplants, but have the advantage of more rapid haematopoietic reconstitution, and in addition can be offered to patients in whom marrow harvest is not feasible due to bone marrow damage or infiltration.

This article reviews the theoretical and historical background to haematopoietic stem cell research, current clinical practice in peripheral blood stem cell mobilisation and harvesting, addresses the potential advantages and disadvantages compared to bone marrow transplantation, and assesses current experience of comparative efficacy.

Haematopoietic stem cells are the progenitors of the haematopoietic and lymphoid systems. They are defined by their facility to give rise to cells of myelomonocytic, erythroid, megakaryocytic and lymphoid lineage by a process of proliferation and differentiation, and possess the capacity to maintain long term haematopoiesis by a process of self renewal.<sup>1</sup>

The rarity of stem cells has led to problems of identification, which have been partially solved by the development of functional assays and immunophenotyping.

## Functional Haematopoietic Stem Cell Assays

The murine spleen colony assay was the first animal model to be developed and is based on the observation

that intravenous injection of bone marrow cells into irradiated mice leads to the development of multilineage colonies in the spleen.<sup>2</sup> These colonies are considered to be the progeny of single clonogenic cells, and are termed colony forming units—spleen (CFU-S). CFU-S can be removed, and re-injected into a second irradiated mouse, where splenic colonies again develop, implying a capacity for self renewal. CFU-S have proved to be heterogeneous in their ability to sustain long term haematopoiesis: CFU-S generated at day +12 after infusion appear to be more primitive than those generated at day +8, and those CFU-S that generate colonies in secondary recipients may be more closely related to pluripotential haematopoietic stem cells with long term repopulating ability.<sup>3</sup>

In man, of course, the CFU-S is not an acceptable assay technique, and several in vitro clonogenic test systems have been developed for the quantitation of haematopoietic progenitors. Lineage-committed stem cells can be assayed in semisolid culture media, and by variation of culture environment, colonies can be

J. I. O. Craig, M. L. Turner, A. C. Parker, Department of Haematology, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh EH3 9YW. Tel: 031 229 2477 Ext 3030, Fax: 031 228 2189.

Correspondence to: M. L. Turner.

induced to form from multipotent cells (colony forming unit—granulocyte, erythroid, monocyte, megakaryocyte, CFU-GEMM) or unipotent cells (colony forming unit—granulocyte, monocyte, CFU-GM; burst forming unit—erythroid, BFU-E; colony forming unit—megakaryocytic, CFU-Meg; colony forming unit—eosinophilic, CFU-Eo). Studies on highly purified populations have suggested that most cells capable of long term repopulation *in vivo*, may not be detected by these kinds of clonogenic assay.<sup>4</sup> Several assays for more primitive stem cells have been developed,<sup>5</sup> these include the human blast cell assay,<sup>6</sup> the human high proliferative potential colony assay<sup>7</sup> and the xenogeneic murine bone marrow transplant model.<sup>8</sup> However, the stem cells giving rise to colonies under these conditions remain poorly characterised.

Recent work suggests that cells capable of forming multilineage colonies on coculture with irradiated long term marrow stromal layers (long term culture—initiating cells),<sup>9</sup> or of surviving *in vitro* 4 hydroperoxycyclophosphamide exposure (pre CFU-GM assay),<sup>10</sup> may be more closely related to the pluripotent haematopoietic stem cell.

At present, the CFU-GM assay is the most widely used measure of the haematopoietic progenitor viability of a bone marrow or PBSC harvest. The number of CFU-GM has been shown to correlate well with restoration of haematopoiesis after bone marrow transplantation in dogs.<sup>11</sup> In man, CFU-GM have been shown to correlate well with neutrophil recovery after bone marrow transplantation, but to a lesser degree with platelet recovery.<sup>12</sup>

### Immunophenotyping of Haematopoietic Stem Cells

The time delay inherent in semisolid culture assays, along with the difficulties in standardisation and reproducibility engendered by variation in culture media, environment and observer error, has led to the application of immunophenotyping and flow cytometry to the problem of stem cell identification. CD34 is a highly glycosylated membrane glycoprotein of unknown function. In man, expression is confined to haematopoietic precursors and vascular endothelial cells. Around 1.5% of low density bone marrow cells are CD34<sup>+</sup>, and virtually all unipotent and multipotent colony forming cells fall within this population.<sup>13</sup> The CD34<sup>+</sup> cell population is heterogeneous. CD34 expression is highest in the earliest progenitors, and is progressively reduced with maturation, such that some of the later progenitors may be operationally defined within the CD34<sup>+</sup> population during analysis by flow cytometry. Pluripotential stem cells capable of forming colonies in long term stromal co-culture and the pre CFU-GM assay, fall within the top 2% of the CD34 fluorescence profile,<sup>9</sup> display low retention of the mitochondrial dye rhodamine 123,<sup>14,15</sup> and lack expression of lineage specific markers.<sup>10,16</sup> Lineage committed stem cells display higher rhodamine 123 retention, and coexpress myeloid (HLA-

DR<sup>+</sup>, CD33<sup>+</sup>),<sup>17</sup> T cell (HLA-DR<sup>+</sup>, TdT<sup>+</sup>, CD7<sup>+</sup>) or B cell (HLA-DR<sup>+</sup>, TdT<sup>+</sup>, CD10<sup>+</sup>, CD19<sup>+</sup>)<sup>18</sup> surface antigens. All subpopulations of CD34<sup>+</sup> cells may be important for rapid and sustained lymphohaematopoietic reconstitution. Several groups have demonstrated a correlation between CD34<sup>+</sup>% and CFU-GM,<sup>19,20</sup> which may allow this antigen to be used as a marker for assessing the viability of a harvest, or to predict peripheral blood stem cell rebound following chemotherapy.<sup>21</sup>

### Peripheral Blood Stem Cells

Under normal circumstances, the majority of haematopoietic stem cells are sessile within the bone marrow, and will preferentially home to the marrow if given by intravenous infusion. Homing mechanisms are, as yet, imperfectly understood, but involve mutual recognition and binding by cell surface adhesion molecules expressed by stem cells, and ligands or counter-receptors expressed in a tissue specific manner by vascular endothelial cells, stromal cells, and the extracellular matrix.<sup>22</sup> Small numbers of haematopoietic stem cells circulate in the peripheral blood in normal individuals. Larger numbers of PBSC can be found under certain pathological and iatrogenic induced conditions discussed below, though whether this arises due to perturbation of the bone marrow microenvironment, or alteration of stem cell adhesion molecule profile, is at present unclear.

### Animal Studies and Early Studies in Man

The observation that buffy coat cells could protect irradiated mice from bone marrow failure was first made in 1951.<sup>23</sup> Transplantation experiments showed that irradiated mice given blood derived allogeneic leukocytes displayed haematopoietic chimerism for several months.<sup>24</sup> At an early stage, the importance of dose of transfused leukocytes was shown in dogs,<sup>25</sup> in which 10 times the number of cells had to be given in order to establish haematological recovery, when compared to bone marrow. When the properties of circulating stem cells were compared to marrow derived stem cells, they were found to have a lower radiosensitivity, with more cells in S phase, and a higher seeding efficiency.<sup>26</sup> Micklem et al.<sup>27</sup> found that peripheral blood and bone marrow stem cells differed markedly in their capacity for self renewal, and the size of descendant populations, suggesting that PBSC represent a selected population with lower long term repopulating ability. However, Northdurft et al.<sup>28</sup> were able to demonstrate long term reconstitution of haematopoiesis in dogs after peripheral blood leukocyte infusion, and showed that colony formation in semisolid culture was a good predictor of engraftment. Harvesting in dogs was facilitated by treatment with cyclophosphamide, which increased the circulating CFU-GM 11-fold, and augmented haematopoietic recovery 12.5-fold after transplan-



tation.<sup>11</sup> These encouraging results in animals led to preliminary work in man.

The presence of colony forming cells in the peripheral blood of man was demonstrated by Chervenick and Boggs in 1971.<sup>29</sup> Kurnick and Robinson<sup>30</sup> cultured peripheral blood leukocytes from patients with viral illnesses with increased numbers of large lymphocytes, and displayed higher levels of granulocytic colonies than the normal population. Barr<sup>31</sup> showed that colony forming activity was displayed predominantly by cells of large lymphocyte morphology.

### Peripheral Blood Stem Cell Mobilisation

Steady state peripheral blood contains approximately  $0.2\% \pm 0.1\%$  CD34<sup>+</sup> cells, about one tenth the level in normal bone marrow ( $1.8\% \pm 0.9\%$ ).<sup>17</sup> Several factors have been shown to increase circulating levels of stem cells 2 to 4-fold, including exercise, ACTH,<sup>32</sup> hydrocortisone,<sup>33</sup> and endotoxin.<sup>34</sup> In certain disease states, particularly myeloproliferative disorders such as myelofibrosis and chronic myeloid leukaemia, greatly increased numbers of circulating progenitors are found.<sup>35-37</sup> The effect of chemotherapy on peripheral blood stem cell levels was first demonstrated by Richman in 1976,<sup>38</sup> who observed a 20-fold increase in circulating CFU-GM over baseline following intermittent chemotherapy for solid tumours. She postulated that it should be possible to obtain sufficient numbers of stem cells for transplantation by judiciously timed leukapheresis. To et al.<sup>39</sup> studied patients with acute myeloid leukaemia (AML) in 1984, and found a 10 to 100-fold increase in CFU-GM in those patients entering remission, which coincided with platelet recovery. Similar increases have been reported in other groups of patients with AML, and also in a small number of patients with acute lymphoblastic leukaemia (ALL).<sup>40,41</sup> We have demonstrated<sup>42</sup> a 5-fold increase in peripheral blood CFU-GM in patients with high grade non Hodgkin's lymphoma (NHL) after routine chemotherapy regimes, and a 16-fold increase following ALL-type therapy. The majority of patients with Hodgkin's disease (HD) receiving routine chemotherapy displayed little or no rise in CFU-GM, though an intensive regime precipitated a 16-fold increase. Bone marrow involvement significantly reduced the peak levels of CFU-GM, as did second or subsequent line chemotherapy. Wunder et al.<sup>43</sup> have demonstrated elevated levels of PBSC after high dose chemotherapy for myeloma.

To et al.<sup>44,45</sup> pioneered the use of single infusions of high dose cyclophosphamide ( $4\text{g}/\text{m}^2$ ) for mobilisation of haematopoietic stem cells in patients with lymphoma, myeloma, and solid tumours. Peak levels of circulating stem cells are reached 16 days after chemotherapy, with mean peak CFU-GM elevated 14-fold above baseline, generally coinciding with the time at which the neutrophil count in the peripheral blood reached  $1 \times 10^9/\text{l}$ . More recently, several groups

have used cyclophosphamide at doses of up to  $7\text{g}/\text{m}^2$ , to achieve greater elevation of CFU-GM.<sup>46,47</sup> Various interrelated factors influence the CFU-GM yield, including the use of higher cyclophosphamide mobilisation doses, higher pre-mobilisation CFU-GM levels, degree of bone marrow involvement, timing and amount of previous chemotherapy, and speed of leukocyte recovery. Unfortunately, such high doses of cyclophosphamide lead to a prolonged period of marrow hypoplasia, with significant associated morbidity and mortality.

In view of the significant toxicity of high dosage cyclophosphamide, and the variable levels of PBSC following standard chemotherapy, several groups have explored the use of recombinant growth factors to mobilise PBSC. In primates, increased numbers of peripheral blood CFU-GM can be induced by recombinant human granulocyte-monocyte colony stimulating factor (rhGM-CSF), interleukin-3 (rhIL-3),<sup>48</sup> and stem cell factor (rhSCF).<sup>49</sup> In man, rhG-CSF used alone produced a 56-fold increase in CFU-GM over baseline,<sup>50</sup> and a 278-fold increase when used in conjunction with standard chemotherapy.<sup>51</sup> rhGM-CSF given alone, or subsequent to chemotherapy, has been shown to increase PBSC levels by a factor of 20 and 60-fold respectively.<sup>52</sup> Gianni et al have demonstrated up to 1000-fold elevation in circulating CFU-GM by the use of high dose cyclophosphamide ( $7\text{g}/\text{m}^2$ ) with rhGM-CSF.<sup>53,54</sup> In phase I studies the use of rhIL-3 alone has proved to be disappointing, with only a 10-fold increase in CFU-GM,<sup>55,56</sup> but recent study has suggested that the sequential use of rhIL-3 and rhGM-CSF may be more effective.<sup>57</sup> Though recombinant growth factors are generally well tolerated, the potential for stimulation of malignant cells remains a concern.<sup>58</sup> On the basis of current experience, we recommend that patients who are potential candidates for PBSC are identified at presentation, and harvested on first line chemotherapy with growth factor support. We harvest after each of at least the first three pulses of chemotherapy, because the yield of PBSC is better early in the course of treatment. Relapsed patients, or those who have failed to achieve good yields of PBSC on standard chemotherapy, can be given high dose cyclophosphamide ( $4\text{g}/\text{m}^2$ ) with growth factors. In patients with residual tumour, the use of cyclophosphamide allows assessment of disease responsiveness before progress to transplantation.

### PBSC Harvesting Techniques

As haematopoietic stem cell activity is found in the 'lymphocyte' fraction of peripheral blood, leukapheresis can be used to harvest progenitors. Many cell separators employing continuous and discontinuous flow have been used successfully such as the Aminco centrifuge,<sup>59</sup> the Hemonetics Model V50,<sup>60</sup> the Cobe Spectra<sup>61</sup> and the Fenwall CS3000.<sup>62</sup> The more recent modifications to protocols, and use of computer

controlled separators have improved the ease and efficiency of the procedure.<sup>63</sup> We employ a Cobe Spectra or a Fenwall CS3000 with a small volume collection chamber, which allows direct cryopreservation of the harvest without further manipulation to reduce the volume or red cell contamination.

Harvests have been cryopreserved with and without further manipulation, mainly with the use of 10% DMSO as a cryoprotectant, and controlled rate freezing with storage in liquid nitrogen at  $-135^{\circ}\text{C}$ .<sup>64,65</sup> It has been suggested that the use of 6% hydroxyethyl starch with 5% dimethyl sulfoxide would reduce stem cell toxicity, whilst allowing direct freezing and storage in a  $-80^{\circ}\text{C}$  freezer.<sup>66</sup>

### Timing of Leukapheresis

The timing and extent of PBSC rebound is difficult to predict on an individual patient basis, and depends upon several factors including the amount of previous chemotherapy or radiotherapy, and the nature of the mobilising agent. The CFU-GM assay requires a 2-week incubation period, which makes day to day monitoring of PBSC levels impossible. Indirect indicators of PBSC mobilisation such as synchronous monocyte and platelet recovery<sup>41</sup> or a rapid rise in white cell count<sup>67</sup> have been advocated by some groups. We currently use a recovering peripheral blood leukocyte count of  $1 \times 10^3/\mu\text{l}$  as a surrogate marker to initiate leukapheresis, and carry out 4 or 5 procedures over subsequent days. Reports from groups finding a close correlation between CD34% and CFU-GM<sup>19,20</sup> raise the possibility of using rapid immunophenotyping to identify early PBSC rebound, allowing early and much more accurate timing of leukapheresis.

### Complications of Bone Marrow Transplantation, and the Potential Advantages of PBSC Transplantation

Bone marrow transplantation (BMT) has found widespread utility in the management of haematological and solid tumours.<sup>68</sup> Allogeneic bone marrow transplantation (AlloBMT) is a major procedure with an overall mortality of 20–50%,<sup>69</sup> related to the complications of prolonged marrow hypoplasia, drug toxicity, and graft versus host disease.<sup>70</sup> A significant limitation is the availability of HLA identical sibling donors in only 35% of the population, and the higher mortality associated with HLA matched unrelated donor transplants.<sup>71</sup>

The use of autologous bone marrow (AutoBMT) overcomes the problem of haematopoietic rescue following high dose chemo-radiotherapy, has more widespread application, and is associated with low risk of graft failure or graft versus host disease. Complications associated with marrow hypoplasia remain a significant cause of morbidity, and are responsible for 10–20% mortality in some reports. Significant disadvantages of AutoBMT include the

potential reinfusion of malignant cells with the harvest, and the loss of a graft versus leukaemia effect, both of which may contribute to a higher rate of disease relapse. Marrow harvest from the posterior iliac crests may not be possible in patients with disease at this site, or in those who have previously received pelvic radiotherapy.

### Peripheral Blood Stem Cell Transplantation

The potential advantages of PBSC over bone marrow autologous transplantation are 3-fold.

Firstly, PBSC can be harvested without the prerequisite of a healthy pelvic bone marrow, and without a general anaesthetic, facilitating this as a therapeutic option for patients with damage to the marrow due to previous disease or radiotherapy. Secondly, rapid haematopoietic reconstitution is seen in those patients transplanted with PBSC collected after mobilisation.<sup>65,72,73</sup> The number of CFU-GM infused needs to be substantially higher ( $80\text{--}100 \times 10^4/\text{kgBW}$ ) than in a bone marrow harvest ( $10\text{--}20 \times 10^4/\text{kgBW}$ ), and correlates well with the rate of granulocyte recovery.<sup>74,75</sup> When adequate numbers of PBSC are infused, a neutrophil count of  $0.5 \times 10^3/\mu\text{l}$  is achieved on average by day +11, and a platelet count of  $50 \times 10^3/\mu\text{l}$  by day +13.<sup>76</sup> This compares favourably with AutoBMT, where neutrophil engraftment occurs at day +22, and platelet engraftment at day +32; and also with AutoBMT augmented by G-CSF, where neutrophil engraftment occurs at day +11, and platelet engraftment at day +39.<sup>50</sup> This translates into a reduction in patient morbidity and resource utilisation.<sup>76</sup> Stem cells collected from peripheral blood in steady state, show recovery rates similar to AutoBMT.<sup>77</sup> Differences in speed of haematopoietic reconstitution may be related to a greater proportion of myeloid-committed stem cells in mobilised PBSC harvests. A recent study by Bender et al<sup>17</sup> has shown a higher coexpression of the myeloid associated antigen CD33 by CD34<sup>+</sup> cells from peripheral blood comparative to those from bone marrow (89 v 43% respectively).

Concern has been voiced from the outset, that PBSC may not contain pluripotential haematopoietic stem cells necessary for long term engraftment. Micklem et al<sup>27</sup> were unable to demonstrate long term repopulating capacity in circulating haematopoietic stem cells in mice. Studies in genetically disparate dogs and primates have confirmed short term engraftment, but the animals usually succumb to graft versus host disease. Most PBSC transplants in man have been autotransplants, and unless the transplanted haematopoietic stem cells are artificially genetically marked, it is impossible to determine the origin of long term post-transplant haematopoiesis. No such studies have yet been reported.<sup>78</sup> Long term recovery of haematopoiesis has been demonstrated in several studies in man,<sup>79,80</sup> but is not necessarily proof of sustained engraftment, because at least some

endogenous haematopoietic stem cells survive chemoradiotherapy,<sup>81</sup> and because initial transient PBSC generated haematopoiesis may be superseded by endogenous recovery. Some support for this thesis comes from the transient fall in granulocytes and platelets sometimes observed about 6 weeks after transplantation,<sup>82</sup> though this can be overcome by infusing larger numbers of CFU-GM. More recently a number of groups have provided support for the presence of pluripotential PBSC on the basis of immunophenotypic characteristics, colony formation in long term stromal cultures,<sup>83</sup> and resistance to 5-Fluorouracil.<sup>84</sup> The timing of leukapheresis may be important in this regard, some studies suggesting that early PBSC recovery is enriched in pluripotent cells, whilst the later phase of mobilisation contains more mature progenitors. These points are important to resolve if PBSC are to be used in an allogeneic setting, or for gene transfer in the future.

A third potential advantage of PBSC transplantation is the differential risk of tumour contamination of the harvest. Several techniques can be used to assess minimal residual disease present in bone marrow or PBSC harvest; these include morphological and cytogenetic analysis,<sup>85</sup> analysis of gene rearrangements by Southern blot and polymerase chain reaction,<sup>86,87</sup> immunological analysis of differentiation antigens,<sup>88</sup> and clonogenic assay.<sup>89</sup> The risk of bone marrow harvest contamination is highest in acute and chronic leukaemias and myeloma, and relatively common in low grade NHL. Marrow involvement occurs less commonly in high grade NHL, HD, and several solid tumours including breast and small cell lung cancer. Several groups have exploited the limited expression of CD34 to explore positive selection of haematopoietic stem cells from contaminated harvest.<sup>90</sup> Such an approach may be expected to effect a 2–4 log removal of neoplastic cells, which may not be sufficient for contaminated bone marrow, but could be sufficient for a PBSC harvest, if contamination is less common or less severe in the latter.<sup>91</sup> Preliminary results do suggest that PBSC are less likely to be contaminated by residual neoplastic cells than bone marrow, but large studies are not yet available, and no consensus has yet been reached as to whether, or how many, infused neoplastic cells may engender relapse. The most common source of relapse after autologous transplantation is likely to be endogenous residual disease which has survived cytotoxic therapy.

### **Comparative Assessment of PBSC and Autologous Bone Marrow Transplantation**

#### *Acute and Chronic Leukaemia*

The role of autologous transplantation in the acute leukaemias has yet to be clearly defined,<sup>92</sup> though in patients without an HLA matched donor, AutoBMT gives an advantage over chemotherapy alone<sup>93</sup> in

poor prognosis disease, or second or subsequent remission.

Adequate reconstitution of haematopoiesis by PBSC in acute myeloid leukaemia (AML) has been demonstrated, Reiffers<sup>94</sup> found a 33% disease free survival 3 years after PBSCT, which is similar to the results of AutoBMT. The European Bone Marrow Transplant Group has not been able to detect any difference in disease free survival between patients receiving PBSCT and AutoBMT.<sup>95,96</sup>

Very few PBSC transplants have been reported in ALL, but two case studies found good engraftment with remission continuing up to 6 months.<sup>97,98</sup>

AlloBMT is the treatment of choice for young patients with chronic myeloid leukaemia (CML).<sup>99</sup> In those patients without a suitable donor, autologous bone marrow harvested in chronic phase can successfully re-establish this phase at the time of acceleration or transformation. However, the duration of second chronic phase is usually short, and the benefit to long term survival is uncertain. Peripheral blood from patients with CML prior to treatment contains large numbers of stem cells, which can be used for transplantation. Some groups have successfully prolonged second chronic phase by double PBSCT ± recombinant alpha interferon.<sup>100</sup> Other groups have successfully restored Philadelphia negative haematopoiesis by collecting Philadelphia negative cells following high dose chemotherapy,<sup>101,102</sup> or by purging the harvest of Philadelphia positive cells by in vitro co-culture with marrow stromal layers. Further work is necessary to establish the durability of such treatments.

#### *Hodgkin's Disease and the Non Hodgkin's Lymphomas*

AutoBMT is used for patients with lymphoma who have proved refractory to front line therapy, or who are in early relapse, or second or subsequent remission.<sup>103</sup>

Kessinger<sup>104</sup> has reported the results of PBSC transplantation in the largest series of patients with lymphoma. 16 patients with NHL and 24 with HD were studied. All had refractory disease and had received much previous therapy. Many had marrow involvement or had received pelvic radiotherapy. The clinical response compared favourably with autologous BMT, with a 2-year event free survival of 49% for those with NHL and an 18 month event free survival of 15% in HD. PBSCT has been successfully used in patients in whom marrow harvest was not possible, in both HD<sup>105–107</sup> and NHL<sup>108–110</sup>, with comparable outcome to AutoBMT.

#### *Multiple Myeloma and Solid Tumours*

AutoBMT has produced some promising results in the management of multiple myeloma, with complete remission of median duration 24 months, though



there is no plateau to the relapse free survival curve,<sup>111</sup> PBSC has proved to be feasible and efficacious in this setting,<sup>112-114</sup> and several groups are evaluating this procedure in the setting of a two phase intensive therapy.<sup>115,116</sup> Experience obtained in breast cancer, soft tissue sarcomas, stage IV neuroblastoma, and recurrent or bulky germ line tumours, suggests that high dose therapy with AutoBMT improves disease free survival.<sup>117,118</sup> Occasional PBSC transplants have been carried out successfully, and are being used increasingly,<sup>119,120</sup> the results of comparative studies are awaited.

### Concluding Remarks

Approximately 500 peripheral blood stem cell transplants have been reported over the last decade, almost all as autotransplants in patients with acute or chronic leukaemia, lymphoma, myeloma or solid tumours. This procedure offers advantages over bone marrow transplantation in patients with marrow disease, and significantly enhances the speed of haematopoietic reconstitution, with less procedure related morbidity and mortality. Despite early worries about the security of long term haematopoietic reconstitution, this does not seem to be a problem in clinical practice, though to what extent this is supported by infused pluripotential stem cells, or by endogenous stem cells which have survived the conditioning regime, is unclear at present. More detailed characterisation of haematopoietic stem cells from different sources, less toxic and more effective mobilising regimes, and more accurate timing of leukapheresis, are areas of prime importance to further improve PBSC transplantation, and to expand its use to the allogeneic setting or for gene therapy. Preliminary evidence of differential levels of minimal residual disease in bone marrow and PBSC harvest, may make this the procedure of choice in some neoplastic diseases. Small trials have shown comparable results between PBSC transplantation and autologous BMT in terms of long term disease free survival. Large scale comparative trials are now necessary to establish the place of this technology in routine clinical practice. It is possible that PBSC transplantation will come to supersede autologous BMT in view of the superior engraftment kinetics, and the ensuing clinical and financial benefit.

### Acknowledgements

Our thanks to the Kay Kendall Leukaemia Trust, the Melville Trust for the Care and Cure of Cancer, and the University of Edinburgh, for financial support.

### References

- Spangrude G J 1991 Haematopoietic stem cell differentiation. *Current Opinion in Immunology* 3: 171-178
- Till J E, McCulloch E A 1961 A direct measurement of the radiation sensitivity of normal bone marrow cells. *Radiation Research* 14: 213-222
- Ploemacher R E, Brons R H 1989 Separation of CFU-S from primitive cells responsible for reconstitution of the bone marrow hemopoietic stem cell compartment following irradiation: evidence for a pre CFU-S cell. *Experimental Hematology* 17: 263-266
- Müller-Sieburg C E, Townsend K, Weissman I L, Rennick D 1988 Proliferation and differentiation of highly enriched mouse hematopoietic stem cells and progenitor cells in response to defined growth factors. *Journal of Experimental Medicine* 167: 1825-1840
- Visser J W M, Van Bekkum D W 1990 Purification of pluripotent hemopoietic stem cells: past and present. *Experimental Hematology* 18: 248-256
- Rowley S D, Sharkis S J, Hattenburg C, Sensenbrenner L L 1987 Culture from human bone marrow of blast cell progenitor cells with an extensive proliferative capacity. *Blood* 69: 804-808
- McNiece I K, Stewart F M, Deacon D M et al 1989 Detection of human CFC with a high proliferative potential. *Blood* 74: 609-612
- Kamel-Reid S, Dick J E 1988 Engraftment of immune deficient mice with human hematopoietic stem cells. *Science* 242: 1706-1708
- Sutherland H J, Eaves C J, Eaves A C, Dragowska W, Lansdorp P M 1989 Characterization and partial purification of human marrow cells capable of initiating long term hematopoiesis in vitro. *Blood* 77: 1563-1570
- Smith C, Gasparetto C, Collins N et al 1991 Purification and partial characterisation of a human hematopoietic precursor population. *Blood* 77: 2122-2128
- Abrams R A, McCormack K, Bowles C, Deisseroth A B 1981 Cyclophosphamide treatment expands the circulating hematopoietic stem cell pool in dogs. *Journal of Clinical Investigation* 67: 1392-1399
- Spitzer G, Verma D S, Fisher R et al 1980 The myeloid progenitor cell—its value in predicting hematopoietic recovery after autologous bone marrow transplantation. *Blood* 55: 317-323
- Civin C I, Trischmann T M, Fackler M J et al 1989. Report on the CD34 cluster workshop. In: Knapp W, Dörken B, Gilks W R et al (eds). *Leukocyte Typing IV: White Cell Differentiation Antigens*. Oxford University Press, Oxford, pp. 818-825
- Bertoncello I, Bradley T R, Hodgson G S, Dunlop J M 1991 The resolution, enrichment, and organisation of normal bone marrow high proliferative potential colony-forming subsets on the basis of rhodamine 123 fluorescence 1991. *Experimental Hematology* 19: 174-178
- Udomsakdi C, Eaves C J, Sutherland H J, Lansdorp P M 1991 Separation of functionally distinct subpopulations of primitive human hematopoietic cells using rhodamine 123. *Experimental Hematology* 19: 338-342
- Andrews R G, Singer J W, Bernstein I D 1990 Human hematopoietic precursors in long term culture: single CD34<sup>+</sup> cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony forming cells when cultured with marrow stromal cells. *Journal of Experimental Medicine* 172: 355-358
- Bender J G, Unverzagt K L, Walker D E et al 1991 Identification and comparison of CD34 positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. *Blood* 77: 2591-2596
- Gore S D, Kastan M B, Civin C I 1991 Normal human bone marrow precursors that express terminal deoxynucleotidyl transferase include T cell precursors and possible lymphoid stem cells. *Blood* 77: 1681-1690
- Siena S, Bregni M, Brando B et al 1991 Flow cytometry for the clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. *Blood* 77: 400-409
- Serke S, Säuberlich S, Huhn D 1991 Multiparameter flow-cytometrical quantitation of circulating CD34<sup>+</sup>-cells: correlation to the quantitation of circulating haematopoietic progenitor cells by in vitro clonal-assay. *British Journal of Haematology* 77: 453-459
- Siena S, Bregni M, Belli N et al 1992 Practical aspects of flow cytometry to guide large-scale collection of circulating hematopoietic progenitors for autologous transplantation in

- cancer patients. *International Journal of Cell Cloning* **10**: 26–29 (Supplement 1)
22. Tavassoli M, Hardy C 1990 Molecular basis of homing of intravenously transplanted stem cells to the marrow. *Blood* **76**: 1059–1070
  23. Brecher B, Cronkite E P 1951 Post-radiation parabiosis and survival in rats. *Proceedings of the Society of Experimental Biology and Medicine* **77**: 292–294
  24. Goodman J W, Hodgson G S 1962 Evidence for stem cells in the peripheral blood of mice. *Blood* **19**: 702–714
  25. Cavins J A, Scheer S C, Thomas E D, Ferrebee J W 1964 The recovery of lethally irradiated dogs given infusions of autologous leukocytes preserved at  $-80^{\circ}\text{C}$ . *Blood* **23**: 38–43
  26. Gidali J, Feher I, Antal S 1974 Some properties of the circulating hemopoietic stem cells. *Blood* **43**: 573–580
  27. Micklem H S, Anderson N, Ross E 1975 Limited potential of circulating haemopoietic stem cells. *Nature* **256**: 41–43
  28. Nordthorft W, Bruch C, Flidner T M, Ruber E 1977 Studies on the regeneration of the CFU-C population in blood and bone marrow of lethally irradiated dogs after autologous transfusion of cryopreserved mononuclear blood cells. *Scandinavian Journal of Haematology* **19**: 470–481
  29. Chervenick P A, Boggs D R 1971 In vitro growth of granulocytic and mononuclear cell colonies from blood of normal individuals. *Blood* **37**: 131–135
  30. Kurnick J E, Robinson W A 1971 Colony growth of human peripheral white blood cells in vitro. *Blood* **37**: 136–141
  31. Barr R D, Whang-Peng J, Perry S 1975 Hemopoietic stem cells in human peripheral blood. *Science* **190**: 284–285
  32. Barrett A J, Longhurst P, Sneath P, Watson J G 1978 Mobilisation of CFU-C by exercise and ACTH induced stress in man. *Experimental Hematology* **6**: 590–594
  33. Morra L, Ponassi A, Parodi G B, Caristo G, Bruzzi P, Sacchetti C 1981 Mobilization of colony-forming cells (CFU-C) into the peripheral blood of man by hydrocortisone. *Biomedicine* **35**: 87–90
  34. Cline M J, Golde D W 1977 Mobilization of haematopoietic stem cells (CFU-C) into the peripheral blood of man by endotoxin. *Experimental Hematology* **5**: 186–190
  35. Chervenick P A 1973 Increase in circulating stem cells in patients with myelofibrosis. *Blood* **41**: 67–71
  36. Partanen S, Ruutu T, Vuopio P 1982 Circulating haematopoietic progenitors in myelofibrosis. *Scandinavian Journal of Haematology* **29**: 325–330
  37. Hibben J A, Nloku O S, Matutes E, Lewis S M, Goldman J M 1984 Myeloid progenitor cells in the circulation of patients with myelofibrosis and other myeloproliferative disorders. *British Journal of Haematology* **57**: 495–503
  38. Richman C M, Weiner R S, Yankee R A 1976 Increase in circulating stem cells following chemotherapy in man. *Blood* **47**: 1031–1039
  39. To L B, Haylock D N, Kimber R J, Juttner C A 1984 High levels of circulating haemopoietic stem cells in very early remission from acute non-lymphoblastic leukaemia and their collection and cryopreservation. *British Journal of Haematology* **58**: 399–410
  40. Bernard Ph, Reiffers J, Vezon G et al 1985 Collection of circulating haemopoietic cells after chemotherapy in acute non-lymphocytic leukaemia. *British Journal of Haematology* **61**: 577–578
  41. Reid C D L, Kirk A, Muir J, Chanarin I 1989 The recovery of circulating progenitor cells after chemotherapy in AML and ALL and its relation to the rate of bone marrow regeneration after aplasia. *British Journal of Haematology* **72**: 21–27
  42. Craig J I O, Smith S M, Parker A C, Anthony R S 1992 The response of peripheral blood stem cells to standard chemotherapy for lymphoma. *Leukaemia and Lymphoma* (in press)
  43. Wunder E, Sovalat H, Liang H, Eisenmann J C, Hénon Ph 1992 Mobilization of peripheral stem cells after high dose chemotherapy in multiple myeloma. *International Journal of Cell Cloning* **10**: 38–40 (Supplement 1)
  44. To L B, Davy M L J, Haylock D N, Dyson P G, Thorp D, Juttner C A 1989 Autotransplantation using peripheral blood stem cells mobilised by cyclophosphamide. *Bone Marrow Transplantation* **4**: 595–596
  45. To L B, Shepperd K M, Haylock D N et al 1990 Single high doses of cyclophosphamide enable the collection of high numbers of hemopoietic stem cells from the peripheral blood. *Experimental Hematology* **18**: 442–447
  46. To L B, Haylock D N, Dyson P G et al 1992 A comparison between  $4\text{g}/\text{m}^2$  and  $7\text{g}/\text{m}^2$  cyclophosphamide for peripheral blood stem cell mobilisation. *International Journal of Cell Cloning* **10**: 33–34 (Supplement 1)
  47. Kotasek D, Shepperd K M, Sage R E et al 1992 Factors affecting blood stem cell collections following high-dose cyclophosphamide mobilization in lymphoma, myeloma, and solid tumours. *Bone Marrow Transplantation* **9**: 11–17
  48. Geissler K, Valent P, Mayer P et al 1992 Mobilization of circulating hemopoietic progenitor cells by rhIL-3 and rhGM-CSF in primates. *International Journal of Cell Cloning* **10**: 47–49 (Supplement 1)
  49. Andrews R G, Bartelmez S H, Knitter G et al 1992 Recombinant human stem cell factor stimulates increased numbers of peripheral blood and marrow CFU-GM, BFU-E, CFU-Mix, HPP-CFC, and CD34<sup>+</sup> cells in baboons. *International Journal of Cell Cloning* **10**: 44–46 (Supplement 1)
  50. Sheridan W P, Begley C J, Juttner C A et al 1982 G-CSF mobilized peripheral and autologous bone marrow infusion following high dose chemotherapy. *International Journal of Cell Cloning* **10**: 71–73 (Supplement 1)
  51. Pettengell R, Demuyneck H, Testa N G, Dexter T M The engraftment capacity of peripheral blood progenitor cells (PBPC) mobilised with chemotherapy  $\pm$  G-CSF. *International Journal of Cell Cloning* **10**: 59–61 (Supplement 1)
  52. Socinski M A, Cannistra S A, Elias A, Antman K H, Schnipper L, Griffin J D 1988 Granulocyte-macrophage colony stimulating factor expands the circulating haemopoietic progenitor cell compartment in man. *Lancet* **i**: 1194–1198
  53. Gianni A M, Siena S, Bregni M et al 1989 Granulocyte-macrophage colony stimulating factor to harvest circulating haemopoietic stem cells for autotransplantation. *Lancet* **ii**: 580–584
  54. Siena S, Bregni M, Brandi B, Ravagnani F, Bonadonna G, Gianni A M 1989 Circulation of CD34<sup>+</sup> hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: enhancement by intravenous human granulocyte-macrophage colony stimulating factor. *Blood* **74**: 1905–1914
  55. Ganser A, Lindemann A, Seipelt G et al 1990 Effects of recombinant human interleukin-3 in patients with normal hematopoiesis and in patients with bone marrow failure. *Blood* **76**: 666–676
  56. Vose J M, Kessinger A, Bierman P J, Sharp G, Garrison L, Armitage J O 1992 The use of rhIL-3 for mobilization of peripheral blood stem cells in previously treated patients with lymphoid malignancies. *International Journal of Cell Cloning* **10**: 62–64 (Supplement 1)
  57. Ottmann O G, Brücher J, Geissler G et al 1992 Effect of sequential treatment with interleukin 3 (rhIL-3) and granulocyte-macrophage colony stimulating factor (rhGM-CSF) on circulating CFU-GM. *International Journal of Cell Cloning* **10**: 65–67 (Supplement 1)
  58. Groopman J E, Molina J M, Scadden D T 1989 Hematopoietic growth factors: biology and clinical applications. *New England Journal of Medicine* **321**: 1449–1459
  59. Korbly M, Flidner T M, Pflieger H 1980 Collection of large quantities of granulocyte/macrophage progenitor cells (CFU-C) in man by means of continuous flow leukapheresis. *Scandinavian Journal of Haematology* **24**: 22–28
  60. Schouten H C, Kessinger A, Smith D M et al 1990 Counterflow centrifugation apheresis for the collection of autologous peripheral blood stem cells from patients with malignancies: a comparison with a standard centrifugation apheresis procedure. *Journal of Clinical Apheresis* **5**: 140–144
  61. Hillyer C D, Tiegerman K O, Berkman E M 1990 Density gradient separation of peripheral blood stem cells: comparison of an automated cell processing device and manual methods. *Transfusion* **30**: 824–827
  62. Lasky L C, Ash R C, Kersey J H, Zanjani E D,

- McCullough J 1982 Collection of pluripotential haematopoietic stem cells by cytopheresis. *Blood* **59**: 822-827
63. To L B, Haylock D N, Thorp D et al 1989 The optimization of collection of peripheral blood stem cells for autotransplantation in acute myeloid leukaemia. *Bone Marrow Transplantation* **4**: 41-47
  64. Gorin N C 1986 Collection, manipulation, and freezing of hemopoietic stem cells. *Clinical Haematology* **15**: 19-48
  65. Korbly M, Martin H 1988 Transplantation of hemapheresis derived hemopoietic stem cells: a new concept in the treatment of patients with malignant lymphohemopoietic disorders. *Plasma Therapy and Transfusion Technology* **9**: 119-132
  66. Kessinger A, O'Kane-Murphy B, Jackson J, Ward B, Kloster T, Dicke K 1992 A comparison of different cryomedia and methods of freezing human hematopoietic stem cells obtained from bone marrow and peripheral blood for storage. *International Journal of Cell Cloning* **10**: 88-91 (Supplement 1)
  67. Cantin G, Marchand-Laroche D, Bouchard M-M, Leblond P-F 1989 Blood-derived stem cell collection in acute non-lymphoblastic leukaemia: predictive factors for a good yield. *Experimental Hematology* **17**: 991-996
  68. Thomas E D 1991 Frontiers in bone marrow transplantation. *Blood Cells* **17**: 259-267
  69. Champlin R, Gale R P 1984 The early complications of bone marrow transplantation. *Seminars in Haematology* **21**: 101-108
  70. Storb R, Prentice R L, Buckner C D et al 1983 Graft versus host disease and survival in patients with aplastic anaemia treated by marrow grafts from HLA-identical siblings. Beneficial effects of a protective environment. *New England Journal of Medicine* **308**: 302-307
  71. Howard M R, Hows J M, Gore S M, Bradley B A for the IMUST study 1991 Unrelated donor marrow transplantation: an interim analysis of the international marrow unrelated search and transplant (IMUST) study II. *Bone Marrow Transplantation* **7**: 126 (Supplement 2)
  72. Bell A J, Hamblin T J, Oscier D G 1986 Circulating stem cell autografts. *Bone Marrow Transplantation* **1**: 103-110
  73. Bell A J, Oscier D G, Figs A, Hamblin T J 1987 Use of circulating stem cells to accelerate myeloid recovery after autologous bone marrow transplantation. *British Journal of Haematology* **67**: 252-253
  74. To L B, Dyson P G, Juttner C A 1986 Cell dose effect in circulating stem cell autografting. *Lancet* **ii**: 404-405
  75. Reiffers J, Leverger G, Marit G et al 1989 Haematopoietic reconstitution after autologous blood stem cell transplantation. In: Gale R P, Champlin R (eds) *Bone Marrow Transplantation: Current Controversies*. Alan R Liss New York, pp. 331-336
  76. Juttner C A, To L B, Roberts M M et al 1989 Comparison of haematological recovery, toxicity, and supportive care of autologous peripheral blood stem cell, autologous and allogeneic bone marrow transplants. *International Journal of Cell Cloning* **10**: 160-161 (Supplement 1)
  77. Kessinger A, Armitage J O, Landmark J D, Smith D M, Weisenburger D D 1988 Autologous peripheral haematopoietic stem cell transplantation following marrow ablative therapy. *Blood* **71**: 723-727
  78. Hénon Ph, Butturini A, Gale R P 1991 Blood-derived haematopoietic cell transplants: blood to blood? *Lancet* **337**: 961-963
  79. Kessinger A, Armitage J O, Smith D M, Landmark J D, Bierman P J, Weisenburger D D 1989 High dose therapy and autologous peripheral blood stem cell transplantation for patients with lymphoma. *Blood* **74**: 1260-1265
  80. Reiffers J, Marit G, Boiron J M 1990 Autologous blood stem cell transplantation in acute leukaemia: present status and future directions. *Bone Marrow Transplantation* **5**: 48-49 (Supplement 1)
  81. Walker H, Singer C R J, Patterson J, Goldstone A H, Prentice H G 1986 The significance of host haematopoietic cells detected by cytogenetic analysis of bone marrow from recipients of bone marrow transplants. *British Journal of Haematology* **62**: 385-391
  82. Juttner C A, To L B, Haylock D N 1990 Approaches to blood stem cell mobilisation. *Initial Australian clinical results. Bone Marrow Transplantation* **5**: 22-24 (Supplement 1)
  83. Bender J G, Unverzagt K L, Walker D E 1992 Characterisation of CD34<sup>+</sup> cells mobilised to the peripheral blood during the recovery from cyclophosphamide chemotherapy. *International Journal of Cell Cloning* **10**: 23-25 (Supplement 1)
  84. Rice A, Reiffers J 1992 Peripheral blood stem cells contain pluripotent cells. *International Journal of Cell Cloning* **10**: 101-107 (Supplement 1)
  85. To L B, Russell J, Moore S, Juttner C A 1987 Residual leukaemia cannot be detected in very early remission peripheral blood stem cell collections in acute non-lymphoblastic leukaemia. *Leukaemia Research* **11**: 327-329
  86. Bell A J, North J, Stevenson F K, Hamblin T J 1990 Peripheral blood stem cell autografts in myeloma. *Bone Marrow Transplantation* **5**: 52-54 (Supplement 1)
  87. Langlands K, Craig J I O, Parker A C, Anthony R S 1990 Molecular determination of minimal residual disease in peripheral blood stem cell harvests. *Bone Marrow Transplantation* **5**: 64-65 (Supplement 1)
  88. Campana D, Coustan-Smith E, Jannossy G 1990 The immunological detection of minimal residual disease in acute leukaemia. *Blood* **76**: 163-171
  89. Aapro M, Irlé C 1988 Cell cultures and detection of minimal residual disease: significance and limitations. *Pathologie Biologie (Paris)* **36**: 56-59
  90. Berenson R J, Bensinger W I, Hill R S et al 1991 Engraftment after infusion of CD34<sup>+</sup> marrow cells in patients with breast cancer or neuroblastoma. *Blood* **77**: 1717-1722
  91. Civin C I 1992 Purified progenitor stem cells: background, feasibility, and prospects for clinical transplantation. *International Journal of Cell Cloning* **10**: 16-19 (Supplement 1)
  92. Gale R P, Butturini A, Reizenstein P 1991 Autotransplants in leukaemia: current state, future prospects. *Leukaemia Research* **15**: 781-784
  93. Mandelli F, Meloni G 1991 The rationale of autologous bone marrow transplantation. *Bone Marrow Transplantation* **7**: 66-67 (Supplement 2)
  94. Reiffers J, Marit G, Boiron J M et al 1990 Autologous blood stem cell transplantation in acute leukaemia: present status and future directions. *Bone Marrow Transplantation* **5**: 48-49 (Supplement 1)
  95. Reiffers J, Korbly M, Labopin M, Hénon Ph, Gorin N C on behalf of the EBMT Group Working Party for Autologous Bone Marrow Transplantation 1992 Autologous blood stem cell transplantation versus autologous bone marrow transplantation for acute myeloid leukaemia in first remission. *International Journal of Cell Cloning* **10**: 111-113 (Supplement 1)
  96. Szer J, Juttner C A, To L B 1992 Post-remission therapy for acute myeloid leukaemia with blood derived stem cell transplantation. Results of a collaborative phase II trial. *International Journal of Cell Cloning* **10**: 114-116 (Supplement 1)
  97. Tilly H, Bastit D, Lucet J-C, Esperou H, Monconduit M, Piguet H 1986 Haematopoietic reconstitution after autologous peripheral blood stem cell transplantation in acute leukaemia (letter). *Lancet* **ii**: 154-155
  98. Bell A J, Figs A, Oscier D G, Hamblin T J 1987 Peripheral blood stem cell autografts in the treatment of lymphoid malignancies: initial experience in three patients. *British Journal of Haematology* **66**: 63-68
  99. Goldman J M 1991 Bone marrow transplantation for chronic myeloid leukaemia. *Bone Marrow Transplantation* **7**: 62-63 (Supplement 2)
  100. Reiffers J, Trouette R, Marit G et al 1991 Autologous blood stem cell transplantation for chronic granulocytic leukaemia in transformation: a report of 47 cases. *British Journal of Haematology* **77**: 339-345
  101. Carella A M, Podestà M, Carlier P, Raffo M R, Pollicardo N, Gualandi F 1992 Conventional intensive therapy can lead to overshoot of Ph negative blood cells in chronic myeloid leukaemia. *International Journal of Cell Cloning* **10**: 117-123 (Supplement 1)
  102. Meloni G, Pinto R M, De Fabritiis P et al 1992 Ablative chemotherapy followed by peripheral blood stem cell reinfusion for chronic myelogenous leukaemia in chronic



- phase. *International Journal of Cell Cloning* 10: 124-126 (Supplement 1)
103. Armitage J O 1989. Bone marrow transplantation in the treatment of patients with lymphoma. *Blood* 73: 1749-1758
  104. Kessinger A, Armitage J O, Smith D M, Landmark J D, Bierman P J, Weisenburger D D 1989. High-dose therapy and autologous peripheral blood stem cell transplantation for patients with lymphoma. *Blood* 74: 1260-1265
  105. Phillips G L, Reece D E, Lerner K G, Connors J M 1990. Intensive chemotherapy and autologous peripheral blood stem cell transplantation in a patient with persistent marrow involvement with Hodgkin's disease. *Bone Marrow Transplantation* 6: 45-47
  106. Kessinger A, Vose J M, Bierman P J, Armitage J O 1992. High dose cyclophosphamide, etoposide and carmustine and peripheral stem cell transplantation for patients with relapsed Hodgkin's disease and bone marrow abnormalities. *International Journal of Cell Cloning* 10: 135-137 (Supplement 1)
  107. Haas R, Hohaus S, Egerer G, Ogniben E, Witt B, Hunstein W 1992. Autologous blood stem cell transplantation in relapsed Hodgkin's disease. *International Journal of Cell Cloning* 10: 138-140 (Supplement 1)
  108. Kessinger A, Armitage J O 1992. Peripheral stem cell transplantation for patients with non Hodgkin's lymphoma. *International Journal of Cell Cloning* 10: 127-128
  109. Kotasek D, Sage R E, Juttner C A, To L B 1992. Autologous transplantation in non Hodgkin's lymphomas using high dose cyclophosphamide mobilised blood stem cells: the Adelaide experience. *International Journal of Cell Cloning* 10: 129-131 (supplement 1)
  110. Majolino I, Quaglietta A M, Iacone A et al 1992. Autologous blood stem cell transplantation in malignant lymphomas; An Italian cooperative study. *International Journal of Cell Cloning* 10: 132-134 (supplement 1)
  111. Barlogie B, Alexanian R, Dicke K A et al 1987. High dosage chemo-radiotherapy and autologous bone marrow transplantation for resistant multiple myeloma. *Blood* 70: 869-872
  112. Bell A J, North J, Stevenson F K, Hamblin T J 1990. Peripheral blood stem cell autografts in myeloma. *Bone Marrow Transplantation* 5: 52-54 (supplement 1)
  113. Fermand J-P, Levy Y, Gerota J et al 1989. Treatment of aggressive multiple myeloma by high-dose chemotherapy and total body irradiation followed by blood stem cell autologous graft. *Blood* 73: 20-23
  114. Marit 1991. Autologous blood stem cell transplantation (ABSCT) in high risk myeloma (MM). *Bone Marrow Transplantation* 7: 29 (supplement 2)
  115. Harousseau J L, Milpied N, Joutet J P 1991. Intensive therapy for high grade multiple myeloma (MM). *Bone Marrow Transplantation* 7: 28 (supplement 2)
  116. Hénou Ph, Eisenmann JC, Beck-Wirth G, Liang H 1992. A two-phase intensive therapeutic approach in high risk myeloma. Follow up. *International Journal of Cell Cloning* 10: 142-144 (supplement 1)
  117. Spitzer G 1991. Autotransplantation for solid tumours. *Blood Reviews* 5: 105-111
  118. Chauvin F, Ladenstein R, Lasset C et al 1991. European Bone Marrow Transplant registry in solid tumours: 7 years experience. *Bone Marrow Transplantation* 7: 157 (supplement 2)
  119. Juttner C A, Davy M L J, To L B et al 1992. Autologous PBSC transplantation in stage 3 and 4 ovarian cancer. *International Journal of Cell Cloning* 10: 145 (supplement 1)
  120. Elias A, Mazanet R, Anderson K et al 1992. GM-CSF mobilised peripheral blood stem cell autografts: the DFCI/BIH experience. *International Journal of Cell Cloning* 10: 149-151 (supplement 1)

# CELL ADHESION MOLECULES: A UNIFYING APPROACH TO TOPOGRAPHIC BIOLOGY

By MARC L. TURNER

*Department of Haematology, Royal Infirmary of Edinburgh, Lauriston Place,  
 Edinburgh EH3 9YW.*

(Received 2 January 1992, accepted 8 June 1992)

## CONTENTS

I. Introduction	359
II. Background remarks	360
III. Molecular biology	360
(1) Cadherin family	361
(2) Immunoglobulin gene superfamily	361
(3) Integrin family	364
(4) Selectin family	365
(5) HCAM (CD44)	365
IV. Illustrations	365
(1) Developmental biology	365
(2) Haemostasis, inflammation and wound repair	367
(3) The immune system: T-cell ontogeny, traffic and function	370
(4) Neoplasia: tumour metastasis and invasion	372
V. Concluding remarks	373
VI. Summary	373
VII. Acknowledgements	374
VIII. References	374

## I. INTRODUCTION

Protozoan organisms exist as discrete entities, or as primitive multicellular aggregates bound together in a non-specific manner (Shapiro, 1988). Metazoan organisms are complex multicellular systems in which tissue structure arises as an emergent property of the interplay between cell differentiation and cell position. A cell's position in the body of the organism is actively determined by the expression of cell surface molecules capable of highly specific binding to molecular ligands on the surface of other cells, or within the extracellular matrix. The pattern of cell adhesion molecule (CAM) expression determines the range of microenvironments within the organism which a particular cell can occupy. Conversely, occupation of such a site through CAM–ligand binding, has a regulatory effect on differential gene expression. A two way process of cell differentiation and cell position is therefore established through cell adhesion molecules, which are pivotal to the development and maintenance of tissue structure in all metazoan organisms.

This article reviews current knowledge of the molecular biology of human cell adhesion molecules, and attempts to provide some illustration of the way in which advances in this field are influencing thinking in human physiology and pathology.

## II. BACKGROUND REMARKS

Close cell-cell contact between mammalian cells is opposed by the negative entropy required for interdigitation of their surface glycocalyces, and mutual negative charge conveyed by sialic acid residues (Bell, Dembo & Bongrand, 1984). Cell-cell and cell-matrix adhesion is therefore an active functional process which involves specific surface membrane proteins termed cell adhesion molecules (CAMs).

Individual CAMs are highly specific in the ligands or counter-receptors to which they bind, in much the same way as cell surface receptors are highly specific in the soluble mediators to which they bind. The mutual binding affinity of a CAM and a particular ligand can be theoretically modelled, and the effects of changes in molecular charge, conformation or surface density assessed (Tozeren, 1990).

Three types of CAM-ligand binding have been described (Alberts *et al.*, 1989).

*Homophilic binding* involves binding of a cell surface molecule to a similar molecule on an apposing cell (*trans*-binding), or sometimes on the adjacent membrane of the same cell (*cis*-binding). Members of the Cadherin family involved in the assembly of adherens and desmosomal junctions, and the Immunoglobulin gene superfamily member NCAM, bind in this way.

Most CAMs, such as Integrin family members, are involved in *heterophilic binding*, i.e. bind to a counter-receptor of different structure on another cell, or to an extracellular ligand such as fibronectin. Members of the Selectin family and HCAM bind to carbohydrate moieties on other cells or within the extracellular matrix. Occasionally, CAMs bind via a *multivalent link molecule*, for example, the binding of platelet gpIIb/IIIa complex to dimeric fibrinogen, which is thought to mediate platelet aggregation.

Most CAMs are transmembrane proteins, consisting of an extracellular domain which is involved in ligand or counter-receptor binding, a hydrophobic transmembrane domain, and an intracellular domain which is often linked to the cytoskeleton. Signal transduction by these molecules is usually bidirectional. The functional differentiation of a cell determines the pattern of CAM expression, but in turn, CAM binding can elicit changes in gene expression and cell phenotype. This property of CAMs, has imbued them with a pivotal role in the development and maintenance of three-dimensional tissue structure in multicellular organisms.

Changes in CAM expression are achieved through alteration in surface expression, or modulation of the binding affinity of a molecule by modification of its chemical structure *in situ*.

During the development of an individual cell lineage the pattern of CAM expression changes allowing association with a variety of other cells and tissues. Individual mature cells may alter expression of CAMs as part of a change in functional status, for example, during activation. Finally, cells may undergo changes in CAM expression patterns during pathological processes such as neoplasia or viral infection. Such changes are not merely passive markers of altered cell phenotype, but a central facet of the physiology of normal and diseased tissue.

## III. MOLECULAR BIOLOGY

There are at least five families of cell surface proteins involved in cell adhesion (Table 1).

Table 1. *Families of cell adhesion molecules*

1. Cadherin family
2. Immunoglobulin gene superfamily
3. Integrin family
4. Selectin family
5. HCAM (CD44)

(1) *Cadherin family*

The Cadherin family includes at least three related molecules involved in calcium dependent, cell-cell homophilic binding (Takaichi, 1988; Takaichi, 1990; Flemming, 1990): E-cadherin (uvomorulin), P-cadherin, and N-cadherin.

Members of the family show similar overall structure, with  $M_r$  124 000, 723–748 amino acids, and 43–58 % mutual homology. They exist as single pass transmembrane glycoproteins, with large extracellular domains which are responsible for homophilic binding. Calcium depletion markedly increases the sensitivity of the molecule to protease degradation. The intracellular domain is linked to the cortical actin cytoskeleton, and is essential to the function of the molecule, allowing concentration of cadherins at junctional complexes. The molecule as a whole links cytoskeletal structures in adjacent cells.

Cadherins are fundamental in establishing and maintaining multicellular structures, especially in the embryonic stages of vertebrate development. Differential expression of these, and other primary CAMs (such as NCAM, see below), allow creation of boundaries between cell collectives destined for different paths of differentiation. Interference in cadherin binding, for example by monoclonal antibody (mAb), gives rise to severe distortion of embryonic topology. In adult tissues, cadherin expression is highest in epithelial derived tissues. Infusion of an anti-E-cadherin mAb over a monolayer culture of epithelial cells, leads to disruption of cell-cell cohesion, breakdown in epithelial sheet, and morphological changes in the epithelial cells, with adoption of a more fibroblastic appearance. It seems very likely that further members of the family will be identified (Goodwin *et al.*, 1990).

(2) *Immunoglobulin gene superfamily*

Members of the Ig gene superfamily (Williams, 1987; Williams, 1988) include receptors which bind counter-receptors or structural ligands, and antigen-specific receptors such as the T-cell receptor (TCR) and surface immunoglobulin (Ig), (Table 2). The molecules of this family are characterized by variable numbers of immunoglobulin domains, which consist of two sheets of anti-parallel  $\beta$ -pleated strands, each containing 90–100 amino acids, and an interdomain disulphide bond. Ligand binding is calcium independent.

The neural cell adhesion molecule (NCAM) was the first member of this family to be isolated and characterized (Cunningham *et al.*, 1987). Structurally it consists of a single pass transmembrane glycoprotein of 1000 amino acids, with five extracellular binding domains which are homologous with those of other members of the immunoglobulin gene superfamily. A single gene codes for NCAM, but differential RNA splicing leads to three major polypeptides which differ in carboxy-terminal structure (transmembrane and cytoplasmic domains). NCAM binding is homophilic,

Table 2. *Major members of the immunoglobulin gene superfamily*

Receptor	Tissue distribution	Ligand	Comments
Neural cell adhesion molecule			
NCAM	Ubiquitous	NCAM	Homophilic
VCAM-1	Vascular endothelial cells	VLA-4	Heterophilic
Intercellular cell adhesion molecule			
ICAM-1 (CD54), ICAM-2		LFA-1	Heterophilic
Lymphocyte function-associated antigens			
LFA-2 (CD2)		LFA-3 }	Mutual counter receptors
LFA-3 (CD58)		LFA-2 }	
Major histocompatibility complex			
Class I	All nucleated cells	CD8 }	Highly polymorphous
Class II	B lymphocytes monocytes/macrophages	CD4 }	
T-cell receptors			
TCR/CD3 complex	All T cells	Antigen + MHC	Somatic rearrangement
CD4	T helper cells	Antigen + Class II MHC	
CD8	T suppressor cells	Antigen + Class I MHC	
Immunoglobulin	B cells (surface and secreted)	Antigen	Somatic rearrangement

and is highly cooperative, i.e. nonlinearly affected by local cell surface density. Indeed at high surface densities *cis*-binding (NCAM–NCAM on the same cell) occurs, leading to a multivalent CAM structure. Modulation of NCAM binding affinity is effected by changes in sialic acid content. Embryonic (E)NCAM has a  $M_r$  200–250 000, and about 30 % sialic acid, Adult (A)NCAM has a  $M_r$  140–180 000, and about 10 % sialic acid. The high negative charge conveyed by sialic acid opposes binding, and it is thought that (E)NCAM has about half the binding affinity of (A)NCAM. The conversion of (E)NCAM to (A)NCAM is thought to be effected by *in situ* enzymatic cleavage, and has major implications in the development of the nervous system (Edelman, 1984).

Like E-cadherin, NCAM is a primary cell adhesion molecule, appearing very early in development, and can be seen at various times on derivatives of all three germ lineages, playing an important role in the development of the central nervous system. Most members of the immunoglobulin gene superfamily are involved in heterophilic binding. Some are mutual counter-receptors, for example the leukocyte function-associated antigens, LFA-2 (CD2) and LFA-3 (CD58) (which, like NCAM, has anchoring isoforms). Others bind to integrin counter-receptors. Intercellular cell adhesion molecule, ICAM-1 (CD54), has five immunoglobulin domains, and is expressed by a wide variety of cells in response to inflammatory mediators. ICAM-2 is structurally very similar, but with only two extracellular immunoglobulin domains, and is constitutively expressed by endothelial cells. Both bind the leukocyte  $\beta_2$  integrin LFA-1. Similarly, vascular cell adhesion molecule, VCAM-1 is expressed on endothelial cells in response to inflammatory stimuli, binding to a monocyte expressed integrin, VLA-4.



Several members of the family play a critical role in the immune system. The Immunoglobulin (Ig) (Tonegawa, 1983) and T-cell receptor (TCR) (Davis & Bjorkman, 1988) genes are unique in that they undergo a process of somatic gene rearrangement in order to generate the vast range of receptor specificity necessary for antigen-specific recognition.

Other members of the family are involved in antigen non-specific immune regulation, these include Major Histocompatibility Complex (MHC) class I and class II molecules (which show a very high degree of genetic polymorphism),  $\beta_2$  microglobulin, CD4 and CD8, poly-Ig receptor, and the Fc $\gamma$  receptor.

Three members of the superfamily are predominantly expressed in the nervous system: NCAM, myelin-associated glycoprotein (MAG), and peripheral myelin glycoprotein (Po). In addition Thy-1 antigen, OX-2, and CD4 are co-expressed both by lymphocytes, and by nervous system tissues. The significance of this is not clearly understood (Parnes & Hunkapiller, 1987; Quarles, Hammer & Trapp, 1990).

Other members of the superfamily include the platelet-derived growth factor receptor (PDGF-R), monocyte colony-stimulating factor receptor (M-CSF R), carcinoembryonic antigen (CEA) (Lance, 1990; Zhou, Fuks & Stanners, 1990) and proteoglycan link proteins.

Proteins with immunoglobulin superfamily homology and cell adhesion functions occur in invertebrates, and it is postulated that an ancient CAM diversified to form the contemporary superfamily, including the molecules responsible for antigen-specific binding within the vertebrate immune systems (Ohno, 1987; Edelman, 1987).

### (3) Integrin family

The integrins comprise a large family of transmembrane glycoproteins with widespread tissue distribution, and involvement in both cell-cell and cell-matrix interaction (Hynes, 1987; Hemler, 1988; Albelda & Buck, 1990). Molecules of this family are heterodimers, with unique  $\alpha$ -subunits, and a small number of  $\beta$ -subunits from which three subfamilies are defined. Subunits are  $M_r$  100–200 000 in size, and considerable structural homology is evident; the various chains are 25–65 % homologous, and the  $\beta$ -chains 37–45 % homologous. The  $\alpha$ - and  $\beta$ -chains of a molecule are non-covalently bound, and both contribute to ligand specificity.  $\alpha$ -Chains demonstrate a highly conserved binding domain to the amino-acid sequence arginine-glycine-asparagine (RGD) (Ruoslahti & Pierschbacher, 1987) which allows many of this family to bind to extracellular matrix (ECM) proteins such as collagen, fibronectin, and laminin. In addition, the  $\alpha$ -chain contains 3–4 repeats of a divalent cation binding site ( $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ). Changes in extracellular calcium concentration affect the topology and functional activity of the *in situ* molecule (Graham & Brown, 1991).

The  $\beta$ -chains are common to subfamilies of receptors, and are bound to the actin-based intracellular cytoskeleton by a series of link proteins which include talin, vinculin and actinin (Alberts *et al.*, 1989). Three main subfamilies are differentiated on the basis of a common  $\beta$ -chain (Table 3).

The  $\beta_1$  subfamily (Hemler, 1990) are termed 'very late activation' because two members (VLA-1 and VLA-2) are expressed on T lymphocytes 2–4 weeks after *in vitro*

Table 3. *Integrin family*

Receptor		Tissue distribution		Ligand
$\beta_1$ (CD29) – VLA (very late activation) family				
$\alpha_1\beta_1$	VLA-1	(CDw49a)	Fibroblasts, basement membrane	Collagen, laminin
$\alpha_2\beta_1$	VLA-2	(CDw49b)	Fibroblasts, epithelial and endothelial cells platelets, T lymphocytes	Collagen, laminin
$\alpha_3\beta_1$	VLA-3	(CDw49c)	Fibroblasts, epithelial cells	Collagen, laminin, fibronectin
$\alpha_4\beta_1$	VLA-4	(CDw49d)	Fibroblasts, monocytes, lymphocytes	Fibronectin, VCAM-1
$\alpha_5\beta_1$	VLA-5	(CDw49e)	Fibroblasts, epithelial and endothelial cells	Fibronectin
$\alpha_6\beta_1$	VLA-6	(CDw49f)	T lymphocytes	Laminin
$\alpha V\beta_1$		(CD51)	Platelets, T lymphocytes Fibroblasts	Fibronectin
$\beta_2$ (CD18) – LEU-CAM (leucocyte cell adhesion) family				
$\alpha L\beta_2$	LFA-1	(CD11a)	All leucocytes	ICAM-1
$\alpha M\beta_2$	MAC-1	(CD11b)	Monocytes, granulocytes	iC3b (inactivated complement 3b)
$\alpha X\beta_2$	p150, 95	(CD11c)	Monocytes, granulocytes	iC3b
$\beta_3$ (CD61) – cytoadhesion family				
$\alpha IIb\beta_3$	gpIIb/IIIa	(CD41)	Platelets	vWF, fibrinogen, fibronectin
$\alpha V\beta_3$	VNR	(CD51)	Endothelial cells, B lymphocytes, monocytes	Vitronectin

The complexity of the family has been increased by the development of novel  $\beta$  subunits which can form alternative complexes with  $\alpha_4$ ,  $\alpha_6$  and  $\alpha V$

activation. The  $\beta_1$  subfamily is widely expressed, and mainly involved in binding to ECM proteins. VLA-4 appears to have dual specificity due to alternative RNA splicing, to the matrix protein fibronectin, and to VCAM-1, an immunoglobulin gene superfamily member expressed by endothelial cells in response to inflammatory mediators.

The  $\beta_2$  subfamily (Larson & Springer, 1990; Arnaout, 1990a) are termed leukocyte cell adhesion molecules, and comprise three members with a common  $\beta_2$  chain (CD18). LFA-1 (CD11a/CD18) is expressed on all leucocytes, and is a counter-receptor for ICAM-1. MAC-1 (CD11b/CD18) and p150,95 (CD11c/CD18) are expressed on phagocytes and large granular lymphocytes, and are receptors for inactivated complement C3b. Genetic deficiency of these molecules leads to poor response to inflammation, and a syndrome of recurrent infections.

The  $\beta_3$  (cytoadhesin) subfamily comprises gpIIb/IIIa, an important platelet adhesion molecule for Fibrinogen, Fibronectin, and von Willebrand Factor (vWF); and the vitronectin receptor (VNR) expressed on endothelial cells and macrophages.

The  $\alpha$ -chains of  $\beta_1$  integrins VLA-1 and VLA-2, and of  $\beta_2$  integrins LFA-1, MAC-1 and p150,95, show homology within binding domains to a number of other proteins, including von Willebrand Factor (vWF), complement components C2 and Factor B, and ECM proteins such as cartilage matrix protein and type VI collagen. These proteins are considered part of a type-A domain superfamily of molecules involved in cell adhesion and extracellular structure (Colombatti & Bonaldo, 1991).

#### (4) *Selectin family*

The Selectin family are C-type lectins, i.e. carbohydrate-binding proteins (Rosen, 1990; Watson *et al.*, 1990; Springer & Lasky, 1991). The N-terminal domain is homologous with a variety of animal lectins, including hepatic galactose receptors, soluble mannose binding lectins, invertebrate lectins, and also the low affinity receptor for IgE (CD23). They are temperature insensitive, and  $\text{Ca}^{2+}$  dependent, though  $\text{Mg}^{2+}$  independent.

There are three members of the family. LECAM-1 (MEL-14/LAM-1) plays a central role in the regulation of lymphocyte traffic. ELAM-1 is expressed on endothelial cells in response to inflammatory stimuli. GMP-140/PADGEM (CD62), is stored in the  $\alpha$  granules of platelets, and in Weibel-Palade bodies in endothelial cells, from where it can be rapidly mobilized after stimulation by products of the clotting cascade.

#### (5) *HCAM (CD44)*

The homing-associated cell adhesion molecule (HCAM) (Haynes *et al.*, 1989; Lewisohn *et al.*, 1989), is a lectin with galactosyl and mannosyl specificity, and is apparently unrelated to other cell adhesion molecules. It displays features related to proteoglycan core proteins, including potential chondroitin sulphate linkage, O-glycosylation sites, and an N-terminal domain homologous to cartilage proteoglycan core and link protein domains involved in glycosaminoglycan interaction. Specificity of binding is achieved through differential splicing of nascent RNA or differential glycosylation of the CD44 molecule. Tissue expression is widespread: the molecule has been identified as of key importance in the recirculation of lymphocytes between blood and lymphoid organs, from which it derives its name.

Carbohydrates are capable of a high degree of structural diversity, and specific protein or lipid bound carbohydrate moieties are expressed on the luminal aspect of endothelial cell surface membrane, often in a tissue specific manner. Such molecules are termed *mucosal vascular addressins*, and are ligands for leukocyte-expressed lectins (in particular LECAM-1 and HCAM), and provide a molecular mechanism for tissue specific traffic of lymphocytes and some neoplastic cells (Berg *et al.*, 1989; Weir, 1989; Brandley, Swiedler & Robbins, 1990).

### IV. ILLUSTRATIONS

#### (1) *Developmental biology*

The development, maintenance and repair of tissue structure (Edelman, 1984; Edelman, 1988; Anderson, 1990; Cunningham, 1991) is dependent on tension between primary cellular driving forces, such as cell growth and proliferation, and cell migration; and primary cell regulatory processes, such as cell adhesion, differential gene expression (differentiation), and programmed cell death (apoptosis) (Wyllie, Kerr & Currie, 1980).

Three-dimensional tissue structure is not directly encoded at the level of the genome, but is an emergent property of interactions occurring at the cellular level, albeit operating under local genetic and epigenetic rules. During the process of embryonic induction (Gordon, 1987), cells of different ontogeny are brought together by

morphogenetic movements with reciprocal effect on cell differentiation, mediated through cell adhesion molecules or receptors for diffusible factors.

Embryonic induction operates mainly on and between cell collectives rather than individual cells, and is a stochastic process. Induction is not necessarily symmetric, for example one cell collective may be primarily induced to differentiate, the other (or several others) acting principally as inducing agents. The central interplay of cell adhesion and cell differentiation during topographic development will be exemplified by two examples: the early processes of gastrulation and neurulation, and the later development of cerebellar mapping.

Following fertilization (Moore, 1988), the zygote undergoes a series of symmetrical mitotic divisions leading to a 12- to 16-cell morula at 3 days. After the morula enters the uterus, a fluid-filled cavity develops inside it, converting the morula into a blastocyst. The centrally localized cells form an inner cell mass, which will form the embryo. From the 1st to the 3rd weeks, the inner cell mass (embryonic disk) is converted into a trilaminar embryonic disk by the process of gastrulation. A groove forms on the dorsal aspect of the embryonic disk (termed the primitive streak). Cells from the superficial layer of the disk migrate through the primitive streak to form a second layer below the first, and a third layer between the first two. From these three primitive germ layers all further tissues will develop. The ectoderm gives rise to the epidermis and nervous systems, the mesoderm: muscles, skeletal structures, the vascular system and urogenital tract, and the endoderm: the primitive gut and its derivatives, including the intra-abdominal organs and lungs.

The process of gastrulation is permitted by the interconversion of cell collectives between two global structural states: epithelium, in which contiguous cells are bound in sheets, with defined polarity and a basement membrane, and mesenchyme, in which the cells are more loosely associated (either stationary or migrating), and with a loose ECM structure (McNeill *et al.*, 1990; Crossin *et al.*, 1990). Interconversion occurs in both directions many times during embryogenesis, and is termed epithelial-mesenchymal transformation.

Between the 3rd and 4th weeks, the embryo undergoes a process of neurulation. Ectodermal tissue anterior to the primitive streak is induced to differentiate by the underlying dorsal mesoderm. A thickened neural plate forms, rolls up on itself and pinches off to form the neural tube, from which the central nervous system will develop. In the middle of the dorsal mesoderm a rod of specialized cells form the notochord, which acts as the central axis of the embryo. On either side of the notochord, lateral segmentation of the mesoderm occurs, with the formation of somites, from which the vertebrae and skeletal muscles develop. Two CAMs have been found to be involved during the earliest stages of embryogenesis: NCAM (Ig gene superfamily), and E-cadherin.

Much of the work on the expression of these two CAMs has been done in chick embryos. Just before gastrulation, cells throughout the embryo express both CAMs fairly uniformly. As the primitive streak develops, cells migrating to form the mesoderm do not express either. As neurulation proceeds, the situation becomes more complex. Cells destined to form the neural plate, notochord, and somites, express NCAM intensely, but stop expressing E-cadherin; the surrounding non-neural ectoderm, and endoderm do the opposite. A border forms between neural and non-

neural ectoderm, which is a direct reflection of the pattern of CAM expression. Furthermore there is a gradient of NCAM expression. Expression is most intense in the region of the neural plate, and less so in underlying mesodermal structures. The pattern of expression is dynamic, for example the notochord at first expresses no CAMs, then expresses NCAM intensely, and finally switches off expression again.

Two generalizations can be made:

(1) CAMs undergo dynamic changes in sequence of appearance, disappearance, and distribution, and permit border formation between different cell collectives.

(2) During mesenchymal to epithelial transformation (condensation), CAM expression is increased, during epithelial to mesenchymal transformation (dispersion), CAM expression is reduced.

Studies at later stages of embryonic development are more restricted. During development of the central nervous system, neurites from peripheral neurons may have to grow substantial distances to form synaptic connection with central neurons. How does a specific peripheral neuron address itself to a specific central neuron?

Early studies on mice (Edelman, 1984) have demonstrated widespread expression of NCAM in the brain, and distinctive patterns of conversion of (E)NCAM to (A)NCAM during development. It appears that NCAM is essential to normal cerebellar development. If NCAM binding is blocked by infusion of monoclonal antibody (mAb) *in vivo*, the mouse develops a markedly hypoplastic cerebellar structure. Moreover, a mutant 'staggerer' mouse has been described, in which the principal abnormality appears to be an inability to convert the E form of NCAM to the more adhesive A form. Failure to form adhesive synapses with incoming neurites, leads to death of cerebellar granule cells, a small and histologically disordered cerebellum, and a severely ataxic mouse.

One can therefore propose a thesis: during cerebellar development (Yamaguchi, 1989; Takaichi *et al.*, 1990) the granule cells form a more or less homogeneous population. Contact with, and adhesion to incoming neurites leads to synapse formation (Yamaguchi, 1989) and differentiation of the granule cell. Cells which do not form such connections undergo apoptosis. The process is instructive, with a high degree of stochastic redundancy, but permits the development of a central cerebellar map without the need for specific molecular addressing of individual cells at the genomic level.

## (2) *Haemostasis, inflammation, and wound repair*

In cybernetic terms, the control of these three processes provides similar and overlapping problems related to the maintenance of tissue integrity. Individual cells exist predominantly in a quiescent state, but need to be activated in response to appropriate stimuli, with changes in cell function, motility, and adhesion. A high degree of stochastic redundancy is usually apparent, in order to provide a rapidly effective response which is not vulnerable to minor component breakdown. Negative control is essential to provide spatial and temporal limitation of the activated response.

Maintenance of the integrity of the vascular system demands rapid and effective control of blood leakage (haemostasis), balanced by the need to maintain vascular patency. Haemostasis is achieved through the combination of vasoconstriction, platelet adhesion, activation and aggregation to form a primary haemostatic 'plug', and activation of the coagulation cascade with generation of polymeric fibrin. This is



followed by tissue remodelling. A complex set of negative feedback systems act to prevent inappropriate platelet activation, to localize coagulation factor activity, and to lyse intravascular thrombus (Kay, 1988).

Circulating platelets do not adhere to intact vascular endothelium under normal circumstances. When a blood vessel is damaged, the endothelial barrier is disrupted, and platelets adhere to the exposed subendothelial surface. The platelet surface membrane has adhesion molecules which bind to specific matrix proteins. These receptors include gpIb/IX complex, a receptor for von Willebrand's Factor (vWF), and several integrin receptors including gpIa/IIa (VLA-2: a collagen receptor), gpIc/IIa (VLA-5: a fibronectin receptor), and gpIc/IIb (VLA-6: a laminin receptor) (Andrews & Fox, 1990; Parmentier *et al.*, 1990). Many components of the matrix such as von Willebrand Factor, thrombospondin, fibronectin and collagen, bind to each other, augmenting the adhesive response, and providing a high degree of redundancy within the system.

Adhesion leads to platelet activation, producing changes in platelet metabolism and morphology, and release of granule contents. These changes act to effect recruitment of more platelets to the haemostatic plug, and to create a local procoagulant microenvironment. The membrane integrin gpIIb/IIIa (Plow & Ginsberg, 1989) is expressed by activated platelets, and binds to fibrinogen and other proteins containing the arginine-glycine-asparagine (RGD) motif. Dimeric fibrinogen molecules probably mediate the aggregation of activated platelets, in association with multimeric vWF.

Platelet procoagulant activity is markedly increased by activation, and the surface membrane expresses binding sites for coagulation proteins. Triggering of the coagulation enzyme cascade leads to formation of multicomponent enzyme complexes on the surface of the developing platelet plug, responsible for the activation of factor X and prothrombin.

Negative control is essential to prevent dissemination of platelet and coagulation cascade activation, and to ensure termination of the response at a suitable juncture. Intact endothelium does not express platelet adhesion counter-receptors, and produces prostacyclin (PGI<sub>2</sub>), a potent anti-aggregant. Unbound activated coagulation proteins are opposed by circulating serine proteinase inhibitors such as antithrombin III, heparin cofactor II, C<sub>1</sub> esterase inhibitor,  $\alpha_1$  antiprotease, and  $\alpha_2$  macroglobulin. Endothelium also expresses surface thrombomodulin, which is activated by thrombin, and binds protein C/S enzyme complex, augmenting the inactivation of coagulation factors V and VIII. In addition endothelium releases tissue plasminogen activator, responsible for activation of circulating plasminogen to form fibrinolytic plasmin. These mechanisms serve to contain the area of thrombus formation.

Genetic deficiencies in platelet adhesion receptors can occur (George & Shattil, 1991): these include Bernard-Soulier disease (gpIb/IX deficiency), and Glanzmann's Thrombasthenia (gpIIb/IIIa deficiency). Clinically these manifest as a bleeding diathesis similar to that caused by thrombocytopenia.

Neutrophils and monocytes circulate in the peripheral blood in a quiescent state. In response to tissue injury, inflammatory mediators are released which cause priming and activation, not only of circulating phagocytes, but also of adjacent endothelium. One or both cell types phenotypically alter to become mutually adhesive (Ward & Marks, 1989; Carlos & Harlan, 1990; Albelda, 1991).

In order to migrate to an inflammatory site, circulating phagocytes must undertake several steps: adherence to the luminal aspect of the endothelium (Patarroyo *et al.*, 1990; Yong & Khwaja, 1990), diapedesis between endothelial cell junctions, migration through the subendothelial extracellular matrix, functional participation in the inflammatory reaction, and programmed senescence.

The primary mediator of activated neutrophil binding to endothelium appears to be CD11a/CD18, though CD11b/CD18 also contributes. In monocytes all three heterodimers of the  $\beta_2$  integrin subfamily are expressed. Constitutive surface expression of these molecules is held at low level. Stimulatory agonists (such as calcium ionophores, phorbol esters, GM-CSF, C5a, tumour necrosis factor (TNF), and an increase in ambient temperature) leads to upregulated surface expression, due to release from intracellular stores. Upregulation is not sufficient in itself to explain the rapid changes in adherence characteristics which occur with phagocyte activation, and it is thought that additional alteration in the biochemical conformation of the surface molecule occurs through phosphorylation, or proteolytic cleavage.

The central role of leukocyte adhesion molecules in the inflammatory response is exemplified by Leukocyte Adhesion Deficiency (LAD) (Fisher *et al.*, 1988; Arnaout, 1990b) in which reduced or absent expression of the CD11/CD18 subfamily on phagocytes, results in an inability to adhere and orientate to the endothelium when stimulated, and failure of emigration in response to inflammatory stimuli. Such patients suffer recurrent severe bacterial infections.

Endothelium constitutively expresses ICAM-1, the counter-receptor for CD11a/CD18 at low level. Upregulation occurs *in vitro* in response to inflammatory mediators such as tumour necrosis factor (TNF-), interleukin 1 (IL-1),  $\gamma$ -interferon ( $\gamma$ IF), phorbol ester and lipopolysaccharide (LPS), and is effected at the mRNA level, being first apparent at 4 h after stimulation, and maximal at about 24 h.

Phagocyte adherence to activated endothelium can be blocked by anti-CD18 mAb *in vitro*, and differences in neutrophil and monocyte adherence can be demonstrated. Other CAMs involved in phagocyte-endothelial cell interactions include ELAM-1, which is not constitutively expressed by endothelial cells, but is rapidly induced by inflammatory stimuli, and which mediates neutrophil binding – though the counter-receptor is unknown. VCAM-1 is expressed in a similar manner, and binds to VLA-4 on monocytes.

Thrombin and leucotriene stimulation also induces endothelial cells to increase ICAM-1 and ELAM-1 expression by increased protein synthesis, and to release GMP-140. The role of these molecules in phagocyte adherence is unknown.

Preliminary studies demonstrate differential sensitivity of phagocytic cells to stimulating agents, and different patterns of CAM expression, which may contribute to differences in kinetic response to inflammatory reaction. Resolution of the inflammatory response involves neutrophil senescence and apoptosis, with aggregation of nuclear chromatin, nucleolar prominence, and cytoplasmic vacuolation. Apoptotic neutrophils are recognized and engulfed by tissue macrophages (Savill *et al.*, 1989), employing the  $\beta_3$  integrin, VNR (Savill *et al.*, 1990).

Although phagocyte activation is an essential mechanism of host defence and tissue repair, it can also contribute significantly to tissue damage through a number of mechanisms, including homotypic intravascular aggregation leading to microvascular

occlusion and ischaemia, release of inflammatory mediators, and neutrophil protease and oxidant release (Editorial, 1990). Neutrophils are thought to play an important role in the pathogenesis of a number of clinical syndromes, including adult respiratory distress syndrome, ischaemia-reperfusion injury following myocardial and cerebral infarction, tissue rejection following transplantation, and vasculitic disorders (Weiss, 1989).

Immunohistological staining for adhesion molecules may prove a useful adjunct to conventional histology in monitoring inflammatory disease. Increased ICAM-1 expression has been demonstrated in a number of conditions including acute and chronic hepatitis (Volpes, van den Oord & Desmet, 1990), allograft rejection (Faull & Russ, 1989), and inflammatory skin conditions (Nickloff, Griffiths & Barker, 1990). Techniques for the assay of circulating ICAM-1 isoforms have also been developed (Seth, Raymond & Makgoba, 1991).

Limited studies have been carried out on the therapeutic use of anti-CAM mAb. Anti-CD11/CD18 mAbs inhibit neutrophil migration in animal models of acute inflammation (Editorial, 1990; Watson, Fennie & Lasky, 1991), and ischaemia-reperfusion injury (Arnaout, 1990a).

The repair process in damaged skin also appears to be mediated by changes in cellular CAM expression and functional phenotype. In normal skin, keratinocytes are relatively static, in wounded skin they undergo changes in cell attachment, with upregulation of  $\alpha_5$  integrin subunits, and structural alteration in  $\beta_1$ -subunit (Grinnel, 1990). Activated keratinocytes undergo spreading and migration in areas of tissue damage and can be cultured *in vitro* for use as wound dressings (Hancock & Leigh, 1989). Fibroblasts similarly undergo changes in CAM expression patterns during wound healing in connective tissues (Chuong & Chen, 1991).

### (3) *The immune system: T-cell ontogeny, traffic and function*

T lymphocytes (Springer, 1990a; Robertson, 1990; Klein, 1991) are derived from pluripotential haematopoietic stem cells (PHSC), which migrate to the embryonic thymus at about 10–14 days' gestation. The thymic stroma, is derived from the embryonic pharyngeal pouch, and is comprised principally of epithelial cells, dendritic cells, and macrophages. Adhesion of PHSC to the thymic microenvironment (Haynes, 1984) induces proliferation and differentiation, with rearrangement of T-cell receptor (TCR)  $\gamma\delta$  genes, followed by TCR  $\alpha\beta$ -gene rearrangement (Finkel, Kubo & Cambier, 1991). This is a process unique to TCR and immunoglobulin (Ig) genes in vertebrates, and is responsible for generating the vast repertoire of binding specificity, characteristic of the immune system. At this stage in development, T cells express both CD4 and CD8 coreceptors. In addition, they express LFA-2, which facilitates binding to thymic epithelial cells, elaborate Interleukin-2 (IL-2), and express LFA-1, which permits binding to ICAM 1 on macrophages, which elaborate IL-1. Other thymic hormones such as IL-4, IL-6, and IL-7, are also elaborated by the thymic stromal cells.

Following generation of large numbers of T cells, with diverse TCR specificity, stringent selection occurs, with both negative and positive selection procedures. Close contact with MHC molecules expressed on thymic epithelial and dendritic cells, leads to apoptosis of those cells incapable of recognizing self MHC, and later, suppression or

elimination of T cells recognizing self antigens in conjunction with self MHC. In the final analysis, T cells capable of recognizing foreign antigen in the presence of self MHC molecules, are selected for maturation into single positive cells (i.e. CD4 + CD8 - or CD4 - CD8 +), and leave the thymus. Apoptotic cells are recognized and removed by tissue macrophages.

Newly emigrated T lymphocytes are considered 'naive' until they encounter and are stimulated by contact with specific antigen, when they are activated, and differentiate into 'memory' cells. The transition from naive to memory phenotype makes the cell more sensitive to TCR binding, and effects permanent changes in surface CAM expression, with alteration in LFA-1, LFA-2, VLA family and HCAM levels. T cells recirculate between blood and lymph, tissues, and secondary lymphoid organs such as lymph nodes and mucosal associated lymphoid tissue (MALT) (Stoolman, 1989; Springer, 1990b; Yednock & Rosen, 1990). Naive and memory T cells display different recirculation patterns (Mackay, Marston & Dudler, 1990), and occupy distinct microenvironments within secondary lymphoid tissues (Janosy *et al.*, 1989). Naive T cells enter lymph nodes from the blood at specialized high endothelial venules (HEV). Memory T cells emigrate from blood through tissue endothelium, and enter local lymph nodes through efferent lymphatics. Memory T cells appear to specifically recognize tissue endothelium similar to that where they first contacted antigen. Such specificity appears to be conveyed by selective expression of carbohydrate based mucosal addressins by endothelial cells from different tissues, recognized and bound by T-cell surface lectins (selectins and HCAM).

As outlined above, the T-cell receptor (TCR) recognizes antigen as peptide fragment bound to Major Histocompatibility Complex (MHC)-coded proteins on the surface of antigen presenting cells. MHC class I molecules are expressed by virtually all nucleated cells, and selectively bind endogenously synthesized peptides. The peptide-MHC class I complex is bound by CD8, expressed on the surface of T suppressor/cytotoxic cells. Cells synthesizing abnormal peptides, e.g. as a result of viral infection, are destroyed. MHC class II molecules are selectively expressed, in particular by B lymphocytes, and monocytes/macrophages, and bind exogenous peptide fragments derived during endocytosis. Peptide-MHC class II complex is bound by CD4, expressed by T helper cells, and leads to the proliferation and maturation of other T cells, and of B cells.

The interaction of T cells with other immune system cells is therefore dependent on two types of cell adhesion molecule, those responsible for antigen specific recognition (i.e. principally TCR/CD3 complex), and those which are antigen non-specific. TCR and its coreceptors (CD4 or CD8) diffuse independently in the plane of the T-cell membrane, and are brought together by corecognition of the same peptide-MHC molecular complex. At physiological densities, no adhesion of T cells will occur. Recognition of peptide-MHC complex, or *in vitro* cross-linking of TCR by mAb, causes T-cell activation, production of amplifying factors - such as IL-2, and upregulation of non antigen specific adhesion molecules. These include LFA-1 (CD11a/18), LFA-2 (CD2), and a series of VLA integrins. The counter-receptors for these molecules (ICAM-1 and LFA-3 respectively), are expressed by a wide variety of cells. T-cell adhesion and detachment needs to occur rapidly, average cycle time is estimated as 1-3 min. Regulation of CAM receptor surface density at the DNA or RNA level, release from intracellular storage, or alteration of surface charge by changes in



sialylation, all occur, but require glycoprotein turnover and *de novo* synthesis and take place over relatively long time scales of 12–24 h. Qualitative changes in CAM function can be achieved over a much shorter time scale. LFA-1 can be converted from a low to a high avidity state by conformational change, controlled by TCR binding via the intracellular cytoskeleton (Dustin & Springer, 1989). Avidity peaks at 5–10 min after TCR binding or cross-linking, and returns to rest over a period of 30 min. In cybernetic terms, LFA-1 acts as a servomotor, under TCR control (Dustin, 1990).

#### (4) *Neoplasia: tumour metastasis and invasion*

In 1889, Stephen Paget published a paper (Paget, 1889) in which he theorized that particular tumours were predisposed to spread to particular sites in the body on the basis of the tissue's ability to support the growth of that tissue. This was known as the 'seed and soil' hypothesis, and seems to have presaged current insight.

Five steps appear critical for successful metastatic spread of a tumour (Zetter, 1990): neoplastic proliferation at the primary site, recruitment of blood vessels (angiogenesis) and intravasation of neoplastic cells, adherence to the endothelium or subendothelial basement membrane at the secondary site, invasion of parenchymal tissue at the secondary site, and proliferation at the secondary site, which is dependent on a favourable microenvironment including growth and mitogenic factors.

The passive trapping of neoplastic cells in pre-capillary vessels is insufficient in itself to permit metastatic spread to a secondary site, site-specific adherence of neoplastic cells to the luminal aspect of the endothelium, or to subendothelial basement membrane is essential (Antonia *et al.*, 1989; Pauli *et al.*, 1990). Experimental studies demonstrate that tumour cells with specific metastatic site preferences, selectively adhere in tissue culture to endothelial cells derived from those preferred sites. Such specificity depends on CAM expression by endothelial cells – presumably mainly carbohydrate addressins, and also adhesive elements of the subendothelial basement membrane (ECM). Evidence suggests that the expression of CAMs by endothelial cells is modulated not only by cytokines, but also by tissue specific components of the ECM (Liotta, 1986; Sher *et al.*, 1988).

Several studies have attempted to correlate alterations in CAM expression with invasive phenotype (Hart, 1990; Mareel, Van Roy & De Baetselier, 1990). Epithelial tumours have proved particularly useful in this regard, because motility of normal epithelial cells is restricted by E-cadherin expression. Well differentiated epithelial tumours, with low invasive potential (I–), show high E-cadherin expression; poorly differentiated tumour with high invasive potential (I+), show low expression. Moreover, invasive phenotype can be converted *in vitro* from I+ to I– by plasmid transfection of cDNA encoding E-cadherin, and from I– to I+ by mAb to E-cadherin, or by plasmid transfection of E-cadherin-specific antisense RNA (Vleminckx *et al.*, 1991). Jouanneau (Thiery *et al.*, 1990) has demonstrated I– to I+ conversion of neoplastic epithelial cells by growth on collagen matrices *in vitro*. The cells undergo downregulation of E-cadherin, with dissolution of desmosomes and intermediary junctions, and assumption of a more motile, fibroblastic appearance, reminiscent of the changes occurring during embryonic epithelial–mesenchymal dispersion.

Immunohistochemical study of CAM expression by neoplastic tissues, promises further insight into tumour classification, management and prognosis (Maio *et al.*,



1990; Horst *et al.*, 1990). It appears clear that normal tissue development and repair processes, and homing mechanisms, provide strong parallels with neoplastic cell behaviour (Pierce & Speers, 1988).

#### V. CONCLUDING REMARKS

This review has attempted to illustrate the way in which advances in our understanding of CAMs are proving a unifying theme in seemingly disparate areas of physiology and pathology. This is not a trivial unification.

The evolutionary emergence of cell-cell adhesion and signalling by way of cell surface molecules, has resulted in place-dependent mechanisms for the control of gene expression. Cell position, as defined by cell adhesion profile, and cell function, as defined by differential gene expression, are interdependent variables. The development and maintenance of tissue structure in metazoan organisms is not directly encoded at the genomic level, but is an emergent property of multicellular interaction. Emergent processes occur when simple agents are connected to each other in complex ways, and are a common feature of many systems – both biological and non-biological (Stein, 1990). All cells are capable of primary driving forces: growth, proliferation, and motility. Metazoan cells are tightly regulated by an interplay between cell differentiation, and cell adhesion/detachment. The rules which govern these processes at the local cellular level, permit the establishment of topology at the level of the cell collective or tissue. The close structural relationship between antigen specific recognition molecules and cell adhesion molecules of the immunoglobulin gene superfamily, is worthy of particular note. The suggestion of a common ancestral origin (Ohno, 1987), implies a close relationship between the immune system and other systems devoted to the maintenance of morphological integrity: haemostasis, inflammation and tissue repair and remodelling. The classic view of the immune system as a defence against foreign invasion may itself be a limited scenario for a system which has evolved in response to a broader imperative – the discrimination of self from aberrant self – cellular morphoregulation.

#### VI. SUMMARY

Cell adhesion molecules are pivotal to the development and maintenance of tissue structure in metazoan organisms. In mammals, several families of proteins are involved in cell-cell and cell-matrix adhesion. The cadherins are homophilic, primary CAMs, involved in the establishment of boundaries between cell collectives early in embryogenesis. The Ig gene superfamily have diversified widely, with homophilic and heterophilic CAMs and antigen recognition molecules amongst the members. The Integrin family play an important role in binding to extracellular matrix, as well as counter-receptors on the surface of other cells. The Selectin family and HCAM are carbohydrate-binding proteins, and play a prominent role in the circulation of lymphocytes and neoplastic cells.

CAMs are fundamental to development of tissue structure in metazoan organisms. Cellular differentiation dictates adherence to a specific microenvironment, through the pattern of surface CAM expression. Conversely, CAM binding can affect gene expression within the cell itself. Cell differentiation and cell adhesion are interdependent processes. In the adult, CAM are crucial to tissue maintenance. Cells frequently change

their adhesive properties in response to physiological or pathological processes. The integrity of the vascular system is maintained by circulating platelets which are capable of rapid upregulation of cell adhesion and profound changes in metabolism, on contact with subendothelial matrix. Both endothelial cells and neutrophils undergo changes in CAM expression in response to inflammatory mediators, permitting rapid and appropriate recruitment of phagocytes to damaged tissue. Tissue repair is dependent on phenotypic changes in normally static cells, allowing increased motility and replication. The immune system requires constitutive cells to undergo multiple complex adhesion and detachment events over short periods of time, and is capable of discriminating normal self from aberrant-self or non-self, through antigen specific recognition and adhesion molecules. The pathophysiology of processes such as infection and neoplasia are profoundly affected by cellular CAM expression.

CAMs and related molecules are fundamental to the development, maintenance and surveillance of tissue structure.

#### VII. ACKNOWLEDGEMENTS

My thanks to Dr H. G. Watson, Dr C. A. Ludlam, Mr T. D. Turner, Dr S. Flemming, Professor C. Haslett, and Dr A. C. Parker, for critical discussion of the manuscript. Thanks to Mr K. Williams for technical assistance. Additional thanks to the Melville Trust for the Care and Cure of Cancer, and the Kay Kendall Leukaemia Trust for financial support.

#### VIII. REFERENCES

- ALBELDA, S. M. & BUCK, C. A. (1990). Integrins and other cell adhesion molecules. *FSAEB Journal* **4**, 2868-2880.
- ALBELDA, S. M. (1991). Endothelial and epithelial cell adhesion molecules. *American Journal of Respiratory Cellular and Molecular Biology* **4**, 195-203.
- ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. & WATSON, J. D. (1989). Cell adhesion, cell junctions, and the extracellular matrix. In: *Molecular Biology of the Cell*, 2nd edn, pp. 792-834. Garland Publishing Inc., New York, London.
- ANDERSON, H. (1990). Adhesion molecules and animal development. *Experientia* **42**, 2-13.
- ANDREWS, R. K. & FOX, J. E. (1990). Platelet receptors in haemostasis. *Current Opinion in Cell Biology* **2**, 821-832.
- ANTONIA, S. J., UCHIDA, J., COHEN, S. & COHEN, M. C. (1989). Attachment of tumour cells to endothelial monolayers: detection of surface molecules involved in cell-cell binding. *Clinical Immunology and Immunopathology* **53**, 281-296.
- ARNAOUT, M. A. (1990a). Structure and function of leukocyte adhesion molecules CD11/CD18. *Blood* **75**, 1037-1050.
- ARNAOUT, M. A. (1990b). Leukocyte adhesion molecule deficiency: its structural basis, pathophysiology, and implication for modifying the immune response. *Immunology Reviews* **114**, 145-180.
- BELL, G. I., DEMBO, M. & BONGRAND, P. (1984). Cell adhesion: competition between nonspecific repulsion and specific binding. *Biophysics Journal* **45**, 1051-1064.
- BERG, E. L., GOLDSTEIN, L. A., JUTILA, M. A., NAKACHE, M., PICKER, L. J., STREETER, P. R., *et al.* (1989). Homing receptors and vascular addressins: cell adhesion molecules that direct lymphocyte traffic. *Immunology Reviews* **108**, 5-18.
- BRANDLEY, B. K., SWIEDLER, S. J. & ROBBINS, P. W. (1990). Carbohydrate ligands of the LEC cell adhesion molecules. *Cell* **63**, 861-863.
- CARLOS, T. M. & HARLAN, J. M. (1990). Membrane proteins involved in phagocyte adherence to endothelium. *Immunology Reviews* **114**, 5-28.
- CHUONG, C. M. & CHEN, H. M. (1991). Enhanced expression of neural cell adhesion molecules and tensin (cytotactin) during wound healing. *American Journal of Pathology* **138**, 427-440.
- COLOMBATTI, A. & BONALDO, P. (1991). The superfamily of proteins with von Willebrand Factor type-A like domains: one theme common to components of extracellular matrix, haemostasis, cellular adhesion and defence mechanisms. *Blood* **77**, 2305-2315.

- CROSSIN, K. L., PRIETO, A. L., HOFFMAN, S., JONES, F. S. & FRIEDLANDER, D. R. (1990). Expression of adhesion molecules and the establishment of boundaries during embryonic and neural development. *Experimental Neurology* **109**, 6-18.
- CUNNINGHAM, B. A., HEMPERLY, J. J., MURRAY, B. A., PREDIGER, E. A., BRACKENBURY, R. & EDELMAN, G. M. (1987). Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* **236**, 799-806.
- CUNNINGHAM, B. A. (1991). Cell adhesion molecules and the regulation of development. *American Journal of Obstetrics and Gynecology* **164**, 939-948.
- DAVIS, M. M. & BJORKMAN, P. J. (1988). T cell antigen receptor genes and T cell recognition. *Nature* **334**, 395-402.
- DUSTIN, M. L. & SPRINGER, T. A. (1989). T cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* **341**, 619-624.
- DUSTIN, M. L. (1990). Two way signalling through the LFA-1 lymphocyte adhesion receptor. *BioEssays* **12**, 21-27.
- EDELMAN, G. M. (1984). Cell adhesion molecules. A molecular basis for animal form. *Scientific American* **250**, 80-91.
- EDELMAN, G. M. (1987). CAMs and Igs: cell adhesion and the evolutionary origins of immunity. *Immunological Reviews* **100**, 11-45.
- EDELMAN, G. M. (1988). *Topobiology: An Introduction to Molecular Embryology*. Basic Books Inc., New York.
- EDITORIAL (1990). Adhesion molecules in the diagnosis and treatment of inflammatory diseases. *Lancet* **336**, 1351-1352.
- FAULL, R. J. & RUSS, G. R. (1989). Tubular expression of intercellular adhesion molecule-1 during renal allograft rejection. *Transplantation* **48**, 226-230.
- FINKEL, T. H., KUBO, R. T. & CAMBIER, J. C. (1991). T cell development and transmembrane signalling: changing biological responses through an unchanging receptor. *Immunology Today* **12**, 79-83.
- FISHER, A., LISOWSKA-GROSPIERRE, B., ANDERSON, D. C. & SPRINGER, T. A. (1988). Leukocyte adhesion deficiency: molecular basis and functional consequences. *Immunodeficiency Reviews* **1**, 39-54.
- FLEMMING, S. (1990). Cellular functions of adhesion molecules. *Journal of Pathology* **161**, 189-190.
- GEORGE, J. N. & SHATTIL, S. J. (1991). The clinical importance of acquired abnormalities of platelet function. *New England Journal of Medicine* **324**, 27-39.
- GOODWIN, L., HILL, J. E., RAYNOR, K., RASZI, L., MANABE, M. & COWIN, P. (1990). Desmoglein shows extensive homology to the cadherin family of cell adhesion molecules. *Biochemical and Biophysical Research Communications* **173**, 1224-1230.
- GORDON, J. B. (1987). Embryonic induction - molecular prospects. *Development* **99**, 281-282.
- GRAHAM, I. L. & BROWN, E. J. (1991). Extracellular calcium results in a conformational change in MAC-1 (CD11b/CD18) on neutrophils. Differentiation of adhesion and phagocytic functions of MAC-1. *Journal of Immunology* **146**, 685-691.
- GRINNEL, F. (1990). The activated keratinocyte: upregulation of cell adhesion and migration during wound healing. *Journal of Trauma* **30**, 5144-5149.
- HANCOCK, K. & LEIGH, I. M. (1989). Cultured keratinocytes and keratinocyte grafts. *British Medical Journal* **299**, 1179-1180.
- HART, I. R. (1990). The invasive phenotype of invasive cells. In: *Cell to Cell Interaction* (ed. M. M. Burger, B. Sordat and R. M. Zinkernagel), pp. 143-151. Karger, Basel.
- HAYNES, B. F. (1984). The human thymic microenvironment. *Advances in Immunology* **36**, 87-142.
- HAYNES, B. F., TELEN, M. J., HALE, L. P. & DENNING, S. M. (1989). CD44. A molecule involved in leukocyte adherence and T cell activation. *Immunology Today* **10**, 423-428.
- HEMLER, M. E. (1988). Adhesive protein receptors on haematopoietic cells. *Immunology Today* **9**, 109-113.
- HEMLER, M. E. (1990). VLA proteins in the integrin family. Structures, functions, and the role on leucocytes. *Annual Reviews in Immunology* **8**, 365-400.
- HORST, E., MEIJER, C. J., RADASZKIEWICZ, T., OSSEKOPPELE, G. J., VAN KRIEKEN, J. H. & PALS, S. T. (1990). Adhesion molecules in the prognosis of diffuse large cell lymphoma: expression of a lymphocyte homing receptor (CD44), LFA-1 (CD11a/CD18) and ICAM-1 (CD54). *Leukaemia* **4**, 383-389.
- HYNES, R. O. (1987). Integrins: a family of cell surface receptors. *Cell* **48**, 549-554.
- JANOSSY, G., BOFILL, M., ROWE, D., MUIR, J. & BEVERLY, P. C. (1989). The tissue distribution of T cells expressing different CD56 phenotypes. *Immunology* **66**, 517-525.
- KAY, L. A. (1988). *Essentials of Haemostasis and Thrombosis*. Churchill Livingstone, Edinburgh.
- KLEIN, J. (1991). *Immunology*. Blackwell Scientific Publications, Oxford.

- LANCE, M. P. (1990). CEA as a cell adhesion molecule. *Gastroenterology* **99**, 277-278.
- LARSON, R. S. & SPRINGER, T. A. (1990). Structure and function of leucocyte integrins. *Immunological Reviews* **114**, 181-217.
- LEWINSOHN, D. M., NAGLER, A., GINZTON, N., GREENBERG, P. & BUTCHER, E. C. (1990). Haemopoietic progenitor cell expression of the H-CAM (CD44) homing-associated adhesion molecule. *Blood* **75**, 589-595.
- LIOTTA, L. A. (1986). Tumour invasion and metastases - role of the extracellular matrix. *Cancer Research* **46**, 1-7.
- MACKAY, C. R., MARSTON, W. L. & DUDLER, C. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. *Journal of Experimental Medicine* **171**, 810-817.
- MAIO, M., PINTO, A., CARBONE, A., ZAGONEL, V., GLOGHINI, A., MAROTTA, G., *et al.* (1990). Differential expression of CD54/intracellular adhesion molecule-1 in myeloid leukaemias and lymphoproliferative disorders. *Blood* **76**, 783-790.
- MAREEL, M. M., VAN ROY, V. M. & DE BAETSELIER, P. (1990). The invasive phenotypes. *Cancer Metastasis Review*, 945-62.
- MCNEILL, H., OZAWA, M., KEMLER, R. & NELSON, W. J. (1990). Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell* **62**, 309-316.
- MOORE, K. L. (1988). *The Developing Human*, 4th edn. W. B. Saunders, Philadelphia.
- NICKOLOFF, B. J., GRIFFITHS, C. E. M. & BARKER, J. N. W. N. (1990). The role of adhesion molecules, chemotactic factors and cytokines in inflammatory and neoplastic skin disease - 1990 update. *Journal of Investigatory Dermatology* **94**, 226-230.
- OHNO, S. (1987). The ancestor of the adaptive immune system was the CAM system for organogenesis. *Experimental and Clinical Immunogenetics* **4**, 181-192.
- PAGET, S. (1889). The distribution of secondary growths in cancer of the breast. *Lancet* **1**, 571-573.
- PARMENTIER, S., KAPLAN, C., CATIMEL, B. & MCGREGOR, J. L. (1990). New families of adhesion molecules play a vital role in platelet functions. *Immunology Today* **11**, 225-227.
- PARNES, J. R. & HUNKAPILLER, T. (1987). L3T4 and the immunoglobulin gene superfamily; new relationships between the immune system and the nervous system. *Immunological Reviews* **100**, 109-128.
- PATARROYO, M., PRIETO, J., RINCON, J., TIMONEN, J., LUNDBERG, C., LINDBOM, L. *et al.* (1990). Leukocyte-cell adhesion: a molecular process fundamental in leukocyte physiology. *Immunology Reviews* **114**, 67-108.
- PAULI, B. U., AUGUSTIN-VOSS, H. G., EL-SABBAN, M. E., JOHNSON, R. C. & HAMMER, D. A. (1990). Organ preference of metastasis. The role of endothelial cell adhesion molecules. *Cancer Metastasis Reviews* **9**, 175-189.
- PIERCE, G. B. & SPEERS, W. C. (1990). Tumours as caricatures of the process of tissue renewal: prospects for therapy by directing differentiation. *Cancer Research* **48**, 1996-2004.
- PLOW, E. F. & GINSBERG, M. H. (1989). Cellular adhesion. GPIIb/IIIa as a prototypic cell adhesion molecule. *Progress in Haemostasis and Thrombosis* **9**, 117-156.
- QUARLES, R. H., HAMMER, J. A. & TRAPP, B. D. (1990). The immunoglobulin gene superfamily and myelination. *Progress in Clinical and Biological Research* **336**, 49-79.
- ROBERTSON, M. (1990). The new biology of immune recognition. *Nature* **34**, 281-282.
- ROSEN, S. D. (1990). The LEC-CAMs: an emerging family of cell-cell adhesion receptors based on carbohydrate recognition. *American Journal of Respiratory Cellular and Molecular Biology* **3**, 397-402.
- RUOSLAHTI, E. & PIERSCHBACHER, M. D. (1987). New perspectives in cell adhesion: RGD and integrins. *Science* **238**, 491-497.
- SAVILL, J. S., WYLLIE, A. H., HENSON, J. E., WALPORT, M. J., HENSON, P. M. & HASLETT, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation. *Journal of Clinical Investigation* **83**, 865-875.
- SAVILL, J. S., DRANSFIELD, I., HOGG, N. & HASLETT, C. (1990). Vitronectin receptor mediated phagocytosis of cells undergoing apoptosis. *Nature* **342**, 170-173.
- SETH, R., RAYMOND, F. D. & MAKGOBA, M. W. (1991). Circulating ICAM-1 isoforms: diagnostic prospects for inflammatory and immune disorders. *Lancet* **338**, 83-84.
- SHAPIRO, J. A. (1988). Bacteria as multicellular organisms. *Scientific American* **258**, 62-69.
- SHER, B. T., BARGATZE, R., HOLZMANN, B., *et al.* (1988). Homing receptors and metastases. *Advances in Cancer Research* **1**, 361-390.
- SPRINGER, T. A. (1990a). Adhesion receptors of the immune system. *Nature* **346**, 425-434.
- SPRINGER, T. A. (1990b). Area code molecules of lymphocytes. In: *Cell to Cell Interaction* (ed. M. M. Burger, B. Sordat and R. M. Zinkernagel), pp. 16-39. Karger, Basel.
- SPRINGER, T. A. & LASKY, L. A. (1991). Cell adhesion. Sticky sugars for selectins (news). *Nature* **349**, 196-197.
- STEIN, D. (ed.). (1990). *Studies in the Sciences of Complexity*. Addison Wesley.
- STOOLMAN, L. M. (1989). Adhesion molecules controlling lymphocyte migration. *Cell* **56**, 907-910.
- TAKAICHI, M. (1988). The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* **102**, 639-655.

- TAKAICHI, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. *Annual Reviews in Biochemistry* **59**, 237-258.
- TAKAICHI, M., INUZUKA, H., SHIMAMURA, K., MATSUNAGA, M. & NOSE, A. (1990). Cadherin mediated cell-cell adhesion and neurogenesis. *Neuroscience Research Supplement* **13**, 892-896.
- THIERY, J. P., DUBAND, J. L., DOFOUR, S., BOYER, B., TUCKER, G. C., VALLES, A. M., GAURILOVIC, J., MOENS, G. & JOUANNEAU, J. (1990). Adhesion and motility of embryonic and cancer cells. *Proceedings of the 6th International Conference on the Differentiation of Normal and Neoplastic Cells*.
- TONEGAWA, S. (1983). Somatic generation of antibody diversity. *Nature* **302**, 575-581.
- TOZEREN, A. (1990). Cell-cell, and cell-substrate adhesion: theoretical and experimental considerations. *Journal of Biomechanical Engineering* **112**, 311-318.
- VLEMINCKX, K., VAKAET, L., MAREEL, M., FIERIS, W. & VAN ROY, F. M. (1991). Genetic manipulation of E-cadherin expression by epithelial tumour cells reveals an invasion suppressor role. *Cell* **66**, 107-119.
- VOLPES, R., VAN DEN OORD, J. J. & DESMET, V. J. (1990). Immunohistochemical study of adhesion molecules in liver inflammation. *Hepatology* **12**, 59-65.
- WARD, P. A. & MARKS, R. M. (1989). The acute inflammatory reaction. *Current Opinion in Immunology* **2**, 5-9.
- WATSON, M. L., KINGSMORE, S. F., JOHNSTON, G. I., SIEGALMAN, M. H., LE BEAU, M. M., LEMONS, R. S., *et al.* (1990). Genomic organisation of the Selectin family of leucocyte adhesion molecules on human and mouse chromosome-1. *Journal of Experimental Medicine* **172**, 263-272.
- WATSON, S. R., FENNIE, C., LASKY, L. A. (1991). Neutrophil influx into an inflammatory site inhibited by a soluble homing receptor-IgG chimera. *Nature* **349**, 164-169.
- WEIR, D. M. (1989). Carbohydrates as recognition molecules in infection and immunity. *FEMS - Microbiology and Immunology* **1**, 331-340.
- WEISS, S. J. (1989). Tissue destruction by neutrophils. *New England Journal of Medicine* **320**, 365-376.
- WILLIAMS, A. F. (1987). A year in the life of immunoglobulin superfamily. *Immunology Today* **8**, 298-303.
- WILLIAMS, A. F. & BARCLAY, A. N. (1988). The immunoglobulin superfamily: domains for cell surface recognition. *Annual Reviews in Immunology* **6**, 381-406.
- WYLLIE, A. H., KERR, J. F. R. & CURRIE, A. R. (1980). Cell death: the significance of apoptosis. *International Reviews in Cytology* **68**, 251-306.
- YAMAGUCHI, K. (1989). Formation of the functional synapse in the cultured nerve cell. *Hum Cell* **2**, 132-136.
- YEDNOCK, T. A. & ROSEN, S. D. (1989). Lymphocyte homing. *Advances in Immunology* **44**, 313-378.
- YONG, K. & KHWAJA, A. (1990). Leucocyte cellular adhesion molecules. *Blood Reviews* **4**, 211-225.
- ZETTER, B. R. (1990). The cellular basis of site-specific tumour metastases. *New England Journal of Medicine* **322**, 605-612.
- ZHOU, H., FUKS, A. & STANNERS, C. P. (1990). Specificity of intercellular adhesion mediated by various members of the immunoglobulin gene superfamily. *Cell Growth and Differentiation* **1**, 209-215.



## HUMAN HAEMATOPOIETIC PROGENITORS AS A TARGET FOR GENE THERAPY

IN JANUARY of this year the Committee on the Ethics of Gene Therapy, chaired by Sir Cecil Clothier, approved the first proposal for the use of gene therapy in the UK, to treat a child with adenosine deaminase deficiency (ADA) by transfection of a normal ADA gene into haematopoietic progenitors.<sup>1</sup> This has been hailed as a ground-breaking therapy, but what are the advantages and pitfalls of haematopoietic progenitors as a target for gene transfection, and what diseases may be amenable to this kind of therapeutic intervention?

### Haematopoietic progenitor cells.

The haematopoietic and lymphoid systems are continuously generated by the proliferation and multilineage differentiation of haematopoietic progenitor cells (HPC). Stem cells form the most primitive subpopulation within the progenitor compartment, capable of extensive self-renewal, pluripotential differentiation and long-term generation of the lymphoid and haematopoietic systems.<sup>2</sup> HPC comprise 1-2% of bone marrow nucleated cells, and a smaller percentage of nucleated cells in adult peripheral blood<sup>3</sup> and umbilical cord blood.<sup>4</sup>

Autologous or allogeneic bone marrow, adult or cord blood can be harvested and stored, and can be used later for "bone marrow" transplantation. Autochthonous lymphohaematopoiesis is usually ablated by high dose chemo-radiotherapy, and the harvest is administered by intravenous infusion. The homing and engraftment of HPC to the marrow stroma is pivotal to the success of this procedure, since the more mature precursors and terminally differentiated cells are short lived. HPC are therefore an attractive target for gene therapy, because they are relatively straightforward to harvest, can be manipulated *in vitro* for gene insertion, and can be returned to the patient by intravenous infusion. Moreover, successful transfection of HPC may lead to the generation of a large self-perpetuating transfected cell mass, theoretically for the life of the recipient.

### Problems with gene transfer into haematopoietic progenitors.

There are several obstacles to the clinical application of gene transfer into HPC. The first is the scarcity of the target cells. Several techniques are now available for *in vitro* production of

enriched HPC populations,<sup>5</sup> including physical methods such as discontinuous density centrifugation and counterflow elutriation, and immunological methods such as panning, immunoaffinity columns, immunomagnetic beads and fluorescence activated cell sorting. Parallel enrichment with physical methods or immunological columns or beads allows preparation of large numbers of cells on the basis of a single parameter. The product is of relatively low purity, but can be used as a platform for multiparameter fluorescence activated cell sorting.<sup>6</sup> A serial protocol of this kind can lead to populations containing >95% HPC, with satisfactory yield and cell viability.

Pluripotent stem cells are a particularly problematic target in that they comprise only 1-2% of the HPC compartment, and are predominantly in quiescent phase.<sup>7</sup> Moreover, stem cells are thought to support lymphohaematopoiesis on the basis of oligoclonal succession; that is, at any one time only a few stem cells actively contribute to the generation of progeny, sequential activation and exhaustion occurs, and effete stem cells are replaced from the quiescent pool.<sup>8</sup> It may therefore be necessary to transfect a high percentage of quiescent stem cells to achieve long-term morphological stability of the derivative population. Quiescent haematopoietic stem cells can be sorted from the progenitor compartment on the basis of high CD34 expression, low rhodamine retention, and low expression of lineage-specific antigens.

Several techniques for cell transfection<sup>9,10</sup> have been developed including physical methods such as calcium phosphate precipitation, lipofusion and electroporation, and viral transfection by retrovirus, SV40, adenovirus, herpes simplex, and adeno-associated virus. Most current protocols employ retroviral vectors<sup>11</sup> which have proved more efficient than other viral or physical methods, but which require target cells to be in cycle to achieve transfection.<sup>12</sup> Considerable progress has been made over the last few years in the *in vitro* manipulation of haematopoietic cells through positive and negative cytokines.<sup>13</sup> Several groups have now provided evidence that incubation of HPC *in vitro* with a combination of cytokines preserves HPC viability and function, and improves retroviral transfection efficiency by recruiting quiescent cells into active proliferation. HPC mobilised to the peripheral blood by the use of *in vivo* chemotherapy and/or growth factors have a particularly high cycling fraction, and may be the optimal source of HPC for retroviral transfer.<sup>3,14</sup> Concerns remain about the possibility of recombination of the retroviral vector, leading to generation of replication competent virus *in vivo*.<sup>15</sup> An alternative approach may be to use a physical method such as lipofusion,<sup>16</sup> which is not

dependent on the cycling status of the target cells, and which avoids the risks associated with biological vectors.

Stable transfection of reporter genes and of several therapeutically relevant human genes into murine and human HPC has now been reported.<sup>11,17</sup> Achieving high levels of sustained expression *in vivo* has proved problematic, however, due to downregulation of gene transcription and competition from autochthonous HPC. Vector constructs containing promotor/enhancer sequences may circumvent this problem, and allow better control over lineage restriction of expression.

### Candidate diseases for HPC transfection

Parkman has proposed a classification of genetic disorders which may be correctable by HPC transplantation, based on the differential expression of the defective gene, and the clinical manifestations of the disorder (Table I).<sup>18</sup> The best candidates for genetic correction are those diseases in which expression of the defective gene is limited to the lymphoid or haematopoietic systems (Classes I-III), or the clinical manifestations are limited to these systems (Class IV) (Table I). HPC transfection may only partially correct the clinical phenotype in Class V diseases such as Lesch-Nyhan syndrome, in which genetic expression and clinical manifestations are generalised. Other Class V defects may be fully correctable if treated early. For example, animal models of Krabbe's disease and fucosidosis can be cured by allogeneic bone marrow transplant, as long as the procedure is carried out within the first few weeks of birth. In man it is now feasible to carry out allogeneic HPC transplantation *in utero*,<sup>19</sup> or within a few weeks of birth, using HPC derived from umbilical cord blood.<sup>4</sup>

Finally, lymphohaematopoietic cells may be engineered to express proteins normally expressed by other tissues (Class VI). HPC may prove a convenient vehicle for gene therapy of some of these disorders, where the clinical manifestations can be corrected by systemic secretion of a normal protein.

Experience in adult allogeneic transplantation has previously suggested that clearance of endogenous HPC by chemotherapy or radiotherapy is a prerequisite for successful engraftment by exogenous HPC. This view has recently been challenged by murine and human studies<sup>19,20</sup> which suggest that partial engraftment can occur in the absence of a preparatory regimen. In many diseases a mixed chimaeric lymphohaematopoietic system may be sufficient to ameliorate the clinical problem. The clinical and technical skills necessary for harvest, *in vitro* manipulation, and reinfusion of genetically manipulated autologous HPC are largely established.

Three therapeutically relevant genes have been transferred into human HPC: the human genes for ADA,  $\beta$  globin, and glucocerebrosidase. The first gene therapy protocols were introduced in September 1990, and involved transfection of peripheral blood T lymphocytes with an ADA gene, in three patients with ADA deficiency.<sup>21</sup> Satisfactory correction of the immune defect has been achieved, but it is unclear how long the effect will last, and infusions of transfected lymphocytes

**Table I**  
Candidate diseases for gene replacement therapy by gene transfer into haematopoietic progenitors

Class I: expression of genetic defect restricted to lymphoid cells non-ADA deficient severe combined immune deficiency X-linked agammaglobulinaemia
Class II: expression restricted to lymphoid and haematopoietic cells. Wiskott-Aldrich syndrome Chediak-Higashi syndrome
Class III: expression restricted to haematopoietic cells $\alpha$ and $\beta$ thalassaemia sickle cell disease and other haemoglobinopathies chronic granulomatous disease platelet storage pool disorders osteopetrosis
Class IV: generalised expression of genetic defect, clinical manifestations restricted to lymphohaematopoietic cells adenosine deaminase deficiency purine nucleoside phosphorylase deficiency Fanconi's anaemia
Class V: generalised expression with generalised clinical manifestations Lesch-Nyhan syndrome (Hypoxanthine-guanine phosphoribosyl transferase deficiency) mucopolysaccharidoses mucopolysaccharidoses eg fucosidosis lipid storage diseases eg: Gaucher's disease (glucocerebrosidase deficiency) Neimann Pick disease (sphingomyelinase deficiency) Krabbe's disease (galactocerebrosidase deficiency)
Class VI: defective gene not normally expressed by lymphohaematopoietic cells. haemophilia A and B (Factor VIII and factor IX deficiency) von Willebrand's disease $\alpha_1$ antitrypsin deficiency.

are to be repeated at yearly intervals. The ADA gene was always expected to be the first candidate for gene therapy because of the relatively small size of the cDNA, and because relatively low levels of expression are sufficient to correct the immune defect. The first clinical studies of ADA transfected HPC have been initiated in Great Ormond Street, London.

Sickle cell anaemia and  $\beta$  thalassaemia are common diseases characterised by defective structure or expression of the  $\beta$  globin gene. Treatment of sickle cell anaemia may require selective removal of the defective  $\beta$  sickle gene, and replacement with a normal  $\beta$  globin gene by homologous recombination.<sup>11</sup> This is not a practical proposition at present.  $\beta$  thalassaemia may be corrected by gene addition, and several reports have described successful transfection of HPC with human  $\beta$  globin gene in mice, though expression has proved to be poor. Inclusion of promoter/enhancer regions may solve this problem, but these elements are large, and their inclusion in retroviral vectors inhibits virus production.

In view of these problems, it is unlikely that the  $\beta$  globin disorders will be the next diseases to be tackled with gene therapy, despite their clinical importance. A more likely candidate is one of the enzyme deficiencies such as Gaucher's disease or the haemophilias, in which lineage restricted expression is not necessarily required, and 10-50% of normal levels of expression should be sufficient to correct the clinical

*phenotype. The gene for human glucocerebrosidase*<sup>9</sup> has been successfully transfected into HPC, but long term expression has not yet been demonstrated. The factor IX gene has been successfully transfected into and expressed by skin fibroblasts,<sup>9</sup> suggesting that appropriate post-translational modification and secretion of a protein can occur in cells other than those normally responsible for production. HPC may prove to be a suitable vehicle for control of the systemic manifestations of these kinds of disease.

Within the next decade, gene therapy is likely to emerge as an important therapeutic modality to correct or modify inherited defects in haematopoiesis, immunity and metabolism.

Haematopoietic progenitors are an obvious target for such intervention, in view of their self-renewing and proliferative potential. Bringing this technology from the laboratory to the clinic is likely to teach us a great deal about the regulation of cellular proliferation and differentiation, the control of gene expression, and the nature of morphological stability in complex regenerative systems.

**M L Turner**

Department of Haematology  
The Royal Infirmary of Edinburgh  
Lauriston Place  
Edinburgh

#### REFERENCES

- 1 Beecham L. Green light for gene therapy. *Medicopolitical Digest*. Br Med J 1993; 306: 658.
- 2 Spangrude GJ. Hematopoietic stem-cell differentiation. *Current Opinion in Immunology* 1991; 3: 171-178.
- 3 Craig JID, Turner ML, Parker AC. Peripheral blood stem cell transplantation. *Blood Reviews* 1992; 6: 59-67.
- 4 Broxmeyer HE, Kurtzberg J, Gluckman E et al. Umbilical cord blood hematopoietic stem and repopulating cells in human clinical transplantation. *Blood Cells* 1991; 17: 313-329.
- 5 Visser JWM, Van Bekkum DW. Purification of pluripotent hemopoietic stem cells: past and present. *Experimental Hematology* 1990; 18: 248-256.
- 6 Weichel W, Irlenbusch S, Kato K, Radbruch A. Sorting of rare cells. In: Radbruch A. *Flow Cytometry and Cell Sorting*. Berlin: Springer-Verlag 1992: 159-167.
- 7 Suda T, Suda J, Ogawa M. Proliferation kinetics and differentiation of murine blast cell colonies in culture: evidence for variable GO periods and constant doubling rates of early pluripotent hematopoietic progenitors. *Journal of Cell Physiology* 1983; 117: 308-318.
- 8 Abkowitz JL, Linebarger ML, Newton MA, Shelton GH, Ott RL, Guttorp P. Evidence for the maintenance of hematopoiesis in a large animal by the sequential activation of stem cell clones. *Proceedings of the National Academy of Sciences USA* 1990; 87: 9062-9066.
- 9 Miller AD. Progress toward human gene therapy. *Blood* 1990; 76: 271-278.
- 10 Larrick JW, Burck KL. *Gene therapy: application of molecular biology*. New York: Elsevier. 1991.
- 11 Karlsson S. Treatment of genetic defects in hematopoietic cell function by gene transfer. *Blood* 1991; 78: 2481-2492.
- 12 Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Molecular Cell Biology* 1990; 10: 4239-4242.
- 13 Moore MAS. Clinical implications of positive and negative hematopoietic stem cell regulators. *Blood* 1991; 78: 1-19.
- 14 Bregni M, Magni M, Siena S, Di Nicola M, Bonadonna G, Gianni AM. Human peripheral blood hematopoietic progenitors are optimal targets of retroviral-mediated gene transfer. *Blood* 1992; 80: 1418-1422.
- 15 Tolstoshev P. Retroviral-mediated gene therapy — safety considerations and preclinical studies. *Bone Marrow Transplantation* 1992; 9: 148-150 (Supp.1)
- 16 Wu GY, Wu CH. Delivery systems for gene therapy. *Biotherapy* 1991; 3: 87-95.
- 17 Dick JE, Kamel-Reid S, Murdoch B, Doedens M. Gene transfer into normal human hematopoietic cells using in vitro and in vivo assays. *Blood* 1991; 78: 624-634.
- 18 Parkman R. The application of bone marrow transplantation to the treatment of genetic diseases. *Science* 1986; 232: 1373-1378.
- 19 Raudrant D, Touraine JL, Rebaud A. In utero transplantation of stem cells in humans: technical aspects and clinical experience during pregnancy. *Bone Marrow Transplantation* 1992; 9: 98-100 (Supp 1)
- 20 Wu DD, Keating A. Hematopoietic stem cells engraft in untreated transplant recipients. *Experimental Hematology* 1993; 21: 251-256.
- 21 Anderson WF. Cautious optimism. (Editorial) *Human Gene Therapy* 1991; 2: 1.

*Reprinted from the Scottish Medical Journal Volume 38 No 5 October 1993*

# STEM CELLS

Volume 12, Number 2, 1994

## **Regulation of Hematopoietic Progenitor Cell Migration, Mobilization and Homing**

*Marc L. Turner*

Department of Haematology, Royal Infirmary of Edinburgh, Edinburgh, Scotland, United Kingdom

*STEM CELLS* 1994;12:227-229

Copyright ©1994 AlphaMed Press. All rights of reproduction in any form

 **AlphaMed Press**

4100 South Kettering Boulevard  
Dayton, OH 45439-2092 (USA)  
☎+513 293-8508  
Fax: +513 293-7652



# Regulation of Hematopoietic Progenitor Cell Migration, Mobilization and Homing

Marc L. Turner

Department of Haematology, Royal Infirmary of Edinburgh, Edinburgh, Scotland, United Kingdom

**Key Words.** Hematopoietic progenitor cells • Cell adhesion

Hematopoietic progenitor cells (HPC) are predominantly sessile within the bone marrow extravascular compartment. During human embryonic and fetal ontogeny, HPC migrate from yolk sac blood islands to the fetal liver and spleen between 15 days and 6 weeks, and to the bone marrow from 20 weeks onwards [1]. Occasionally, HPC migration can occur between dizygotic twins in utero through placental vascular anastomoses, leading to chimeric hematopoiesis [2]. The mechanisms underlying this sequential migration are not understood [3]. At birth significant numbers of HPC are present in umbilical cord blood and these can be harvested and used for allogeneic transplantation [4]. In healthy adults, HPC migrate from the bone marrow to other tissues including the spleen, thymus and areas of mast cell development in the stomach, cecum, mesentery and skin [5]. Areas of the bone marrow which have been naturally or artificially damaged can be recolonized by migration from healthy marrow [6]. Low levels of HPC circulate in the peripheral blood of adults, but a substantial rise can occur under a variety of physiological, pathological, and iatrogenic conditions. These cells can be harvested by leukapheresis and are finding widespread use in clinical transplantation [7]. Following high dose chemotherapy with or without radiotherapy, ablated marrow can be recolonized by intravenous infusion of HPC from autologous or allogeneic bone marrow, cord blood or peripheral blood. Homing and

engraftment of HPC to the bone marrow are thought to be mediated through HPC expression of cytoadhesion molecules with high specificity recognition of, and adhesion to, ligands or counter-receptors expressed by the luminal aspect of the sinusoidal endothelium and stromal cells and extracellular matrix [8]. HPC migration, mobilization and homing are related phenomena, and although considerable progress has been made in our understanding of cytoadhesion mechanisms at the molecular level, a unified theory of the regulation of these processes has yet to be established. Two approaches may be used to construct such a theory.

## A Deterministic Model of HPC Migration and Mobilization

A deterministic model proposes that progenitor migration and mobilization are instructed at the cellular level by an intrinsic program or an extrinsic cybernetic system. Migration is understood as a teleological phenomenon [9], HPC egress the marrow in response to a specific stimulus, possibly a homeostatic feedback signal, and are directed to a specific target tissue.

During fetal ontogeny HPC undergo sequential migration. A deterministic model of migration postulates developmental stage-specific modulation of cytoadhesion molecule expression to permit sequential detachment, circulation and homing to a new hematopoietic environment. Modulation of cytoadhesion molecule expression must coincide with maturation of the appropriate environment, implying precise developmental timing or signaling from the maturing target tissue to the site of primary hematopoiesis.

Correspondence: Dr. Marc L. Turner, Department of Haematology, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh, Scotland, United Kingdom.

Received May 20, 1993; provisionally accepted July 5, 1993; accepted for publication September 30, 1993. ©AlphaMed Press 1066-5099/94/\$5.00/0



In the normal adult, HPC undergo migration from the bone marrow to various tissues, including the thymus and spleen. Partial body irradiation experiments in mice have shown that damaged marrow can be repopulated by HPC migrating from a protected area [6], and that this is accompanied by a decline in HPC in the shielded marrow [10]. It is unclear as to whether this is a reflection of migration of HPC in response to humoral feedback communication between depleted and normal marrow, or an increased rate of HPC differentiation in response to depletion of committed precursors [10, 11]. If HPC do undergo tissue-specific migration in response to humoral feedback, then similar cybernetic mechanisms may be necessary to regulate migration to other lymphohematopoietic tissues.

The interpretation of HPC mobilization proves difficult within this theoretical framework. A wide variety of agents can cause elevation of peripheral blood HPC levels [7], including exercise, adrenocorticotrophic hormone (ACTH), hydrocortisone, endotoxin, dextran sulphate, chemotherapy and recombinant growth factors.

Two possible explanations may be offered: first, that HPC downregulate cytoadhesion molecule expression in response to a wide variety of stimuli, through mechanisms which have yet to be elucidated. If so, it becomes difficult to explain the rapid engraftment of peripheral blood and umbilical cord blood derived progenitors following transplantation. Second, damage to the blood/marrow interface or elements of the marrow stroma may allow uncontrolled egress of normal HPC. *Tavassoli* has provided evidence of breakdown in the functional integrity of the marrow endothelium in response to irradiation, and has suggested that this preempts endothelial recognition and permits unregulated transmigration post-transplant [12]. However, one has to argue for a preferential sensitivity of marrow endothelium to chemo/radiotherapy, or a generalized breakdown in endothelial function which may lead to indiscriminate homing. It seems unlikely that endothelial or stromal damage can be held responsible for the rapid HPC mobilization observed following exercise or administration of dextran sulphate, or for the delayed mobilization seen during recovery from high-dose chemotherapy. Different mechanisms of homing and mobilization may, of course, predominate under different circumstances.

### A Stochastic Model of HPC Migration and Mobilization

A stochastic model proposes that HPC migration and mobilization are emergent features of HPC population dynamics and are a consequence of physical rather than teleological causation [8]. The pattern of tissue migration and homing is understood to be the result of selection of circulating HPC through competitive binding to available niches. This model proposes that constitutive overproduction of HPC leads to the presence of homeless cells, which "wander" within the bone marrow and egress into the peripheral blood. If adherence of HPC to stroma is a reversible process, the relative avidity of which is determined by the pattern of cellular cytoadhesion molecule expression, then there may be ongoing competition for available niches and a dynamic equilibrium between incumbent and circulating HPC. Recent work has demonstrated that infused HPC can engraft bone marrow in the absence of a conditioning regimen in immune deficient mice [13], and also in the immunologically immature human fetus [14]. *Crittenden et al.* [15] have demonstrated that repeated marrow transfusion in non-myeloablated mice gives rise to sequential incremental increases in the percentage of allogenic engrafted HPC and have suggested that approximately 10% of incumbent (host) HPC may be replaced following each infusion. Modest overproduction provides an explanation for the presence of HPC in the peripheral blood of healthy individuals, and colonization of new microenvironmental niches in developing fetal hematopoietic tissues, and local and remote niches in adult spleen, thymus or gastrointestinal hematopoietic tissues, or in damaged bone marrow. Moreover, the pool of circulating HPC may act as a buffer, ensuring engraftment of all potential microenvironmental niches and maintaining consistent support of the hematopoietic and lymphoid systems. HPC unable to find a suitable microenvironmental home probably undergo apoptosis due to a lack of growth factor support [16], leading to a selective but redundant method of maintaining a stable HPC population.

Elevation of peripheral blood HPC may arise within this framework through several mechanisms. The size of the circulating HPC population can be postulated to be dependent on

the proliferative rate of HPC, the extent of dynamic interchange between incumbent and circulating cells, and the survival capacity of HPC in the absence of stromal support. Different hematopoietic tissues may provide a different proliferative stimulus, or different adhesion kinetics. For example, individuals in whom the spleen is a major organ of hematopoiesis (e.g., neonates), have elevated levels of circulating HPC which may be due to a more rapid dynamic interchange between sessile and circulating cells, allowing support of a larger circulating population.

In adults, "mobilization" may occur in response to demargination (e.g., exercise, ACTH, hydrocortisone or endotoxin), blockage of HPC-endothelial adhesion (e.g., dextran sulphate), rebound overproduction of HPC in response to temporary marrow aplasia (e.g., high dose chemotherapy), or protection against apoptosis (e.g., growth factors [14]). Different mechanisms acting contemporaneously (e.g., chemotherapy and growth factors) may act synergistically, giving rise to a particularly high and sustained level of circulating HPC.

Neither of these theoretical approaches currently provides a comprehensive explanation of hematopoietic progenitor migration and mobilization, nor are they necessarily mutually incompatible. Areas of confrontation should prove informative fields for experimental scrutiny.

## Acknowledgments

My thanks to *Dr. John Ansell* and *Dr. John Sweetenham* for critical review of this manuscript.

## References

- 1 Tavassoli M. Embryonic and fetal hemopoiesis: An overview. *Blood Cells* 1991;17:269-281.
- 2 Tippet P. Blood group chimaeras: A review. *Vox Sanguinis* 1983;44:333-359.
- 3 Broxmeyer HE. Self-renewal and migration of stem cells during embryonic and fetal hematopoiesis: Important but poorly understood events. *Blood Cells* 1991;17:282-286.
- 4 Broxmeyer HE, Gluckman E, Auerbach A, Douglas GW, Friedman H, Cooper S, Hangoc G, Kurtzberg J, Bard J, Boyse EA. Human umbilical cord blood: A clinically useful source of transplantable hematopoietic stem/progenitor cells. *Intl J Cell Cloning* 1990;8:76-91.
- 5 Trentin JJ. Hemopoietic microenvironments: historical perspectives, status and projections. In: Tavassoli M, ed. *Handbook of the Hemopoietic Microenvironment*. New Jersey: Humana Press. 1989:1-78.
- 6 Hanks GE. In vivo migration of colony forming units from shielded bone marrow in the irradiated mouse. *Nature* 1964;203:1393-1395.
- 7 Craig JIO, Turner ML, Parker AC. Peripheral blood stem cell transplantation. *Blood Reviews* 1992;6:59-67.
- 8 Tavassoli M, Hardy C. Molecular basis of homing of intravenously transplanted cells to the marrow. *Blood* 1990;76:1059-1070.
- 9 Medawar PB. D'Arcy Thompson and "Growth and Form." In: *Pluto's Republic*. Oxford: Oxford University Press. 1988:228-241.
- 10 Gidali J, Lajtha LG. Regulation of haemopoietic stem cell turnover in partially irradiated mice. *Cell Tissue Kinetics* 1972;5:147-157.
- 11 Lord BI. Haemopoietic stem cells. In: Potten CS, ed. *Stem Cells: Their Identification and Characterization*. Edinburgh: Churchill Livingstone, 1983:118-154.
- 12 Tavassoli M. The role of conditioning regimens in homing of transplanted hemopoietic cells. *Bone Marrow Transplant* 1992;10:15-17.
- 13 Wu DD, Keating A. Hematopoietic stem cells engraft in untreated transplant recipients. *Exp Hematol* 1993;21:251-256.
- 14 Raudrant D, Touraine JL, Rebaud A. In utero transplantation of stem cells in humans: Technical aspects and clinical experience during pregnancy. *Bone Marrow Transplant* 1992;9(1):98-100.
- 15 Crittenden R, Rao S, Quesenberry PJ. Repetitive marrow transplantation: A model for autologous gene therapy. *Exp Hematol* 1993;21:1016a.
- 16 Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor DA. Haemopoietic colony-stimulating factors promote cell survival by suppressing apoptosis. *Nature* 1990;343:76-79.

# STEM CELLS

Volume 13, Number 2, 1995

## **Differential Expression of Cell Adhesion Molecules by Human Hematopoietic Progenitor Cells from Bone Marrow and Mobilized Adult Peripheral Blood**

*Marc L. Turner,<sup>a</sup> Kathleen McIlwaine,<sup>c</sup> Robert S. Anthony,<sup>b</sup> Alistair C. Parker<sup>b</sup>*

Departments of <sup>a</sup>Transfusion Medicine and <sup>b</sup>Haematology, Royal Infirmary of Edinburgh and <sup>c</sup>Department of Haematology, Western General Hospital, Edinburgh, Scotland, United Kingdom

STEM CELLS 1995;13:311-316

Copyright ©1995 AlphaMed Press. All rights of reproduction in any form reserved.

For subscription order information, please contact:

 AlphaMed Press

4100 South Kettering Boulevard  
Dayton, OH 45439-2092 USA

☎ 513 293-8508

Fax: 513 293-7652

# Differential Expression of Cell Adhesion Molecules by Human Hematopoietic Progenitor Cells from Bone Marrow and Mobilized Adult Peripheral Blood

Marc L. Turner,<sup>a</sup> Kathleen McIlwaine,<sup>c</sup> Robert S. Anthony,<sup>b</sup> Alistair C. Parker<sup>b</sup>

Departments of <sup>a</sup>Transfusion Medicine and <sup>b</sup>Haematology, Royal Infirmary of Edinburgh and <sup>c</sup>Department of Haematology, Western General Hospital, Edinburgh, Scotland, United Kingdom

**Key Words.** Hematopoietic progenitor cells • CD34 • Cell adhesion molecules

**Abstract.** The mechanisms responsible for mobilization of hematopoietic progenitor cells (HPC) from the bone marrow into the circulation are unknown. One possibility is that HPC downregulate cell adhesion molecule expression. We studied normal human bone marrow and adult peripheral blood following 4g/m<sup>2</sup> cyclophosphamide and recombinant human granulocyte colony-stimulating factor (rHuG-CSF). Each sample was studied for the coexpression of CD34 and a panel of cell adhesion molecules by dual immunocytometry. Bone marrow HPC express the immunoglobulin gene superfamily members ICAM-1 (CD54), PECAM-1 (CD31) and LFA-3 (CD58), the integrins VLA-4 (CD49d/CD29), VLA-5 (CD49e/CD29) and LFA-1 (CD11a/CD18), L-Selectin (CD62L), HCAM (CD44) and CD36. Mobilized peripheral blood HPC display less expression of LFA-3 (CD54) and VLA-5 (CD49e). Significant differences in cell adhesion molecule expression do exist between sessile and circulating HPC, but the biological relevance of these observations is currently unclear.

## Introduction

Hematopoietic progenitor cells (HPC) are predominantly sessile within the bone marrow but also circulate at low levels in the peripheral blood, migrate between hematopoietic tissues during fetal development and adult life [1],

and home to the marrow following intravenous infusion [2]. Elevation of peripheral blood HPC occurs under a variety of physiological, pathological and iatrogenic conditions. HPC harvested by leukapheresis from the peripheral blood of patients with neoplastic disease during the recovery phase following chemotherapy and/or recombinant growth factors can be used for transplantation [3]. The mechanisms by which HPC are released into the peripheral blood, circulate and home to specific tissues are poorly understood [1, 4]. One of several possibilities [5] is that alteration in HPC cell adhesion molecule expression or function is responsible for HPC egress from the marrow.

We have used dual immunocytometry to study the comparative expression of cell adhesion molecules by the CD34<sup>+</sup> HPC population from normal human bone marrow and from adult peripheral blood following high dose cyclophosphamide and recombinant human granulocyte colony-stimulating factor (rHuG-CSF). We focused on those cell adhesion molecules which other groups have described as expressed by bone marrow HPC: the immunoglobulin gene superfamily members ICAM-1 [6, 7] (CD54), PECAM-1 [8, 13] (CD31) and LFA-3 [7] (CD58), the integrins VLA-4 (CD49d/CD29), VLA-5 (CD49e/CD29), LFA-1 (CD11a/CD18) and VNR [7, 9-12] (CD51/CD61), L-Selectin [13] (CD62L), HCAM [7, 11, 14] (CD44) and CD36 [15, 16] (Table I). There is little evidence that other adhesion molecules of the immunoglobulin, integrin or selectin families are expressed by bone marrow HPC [7, 9, 10].

Correspondence: Dr. Marc Turner, Department of Transfusion Medicine, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh EH3 9HB, Scotland, UK.

Received December 8, 1994; provisionally accepted January 10, 1995, accepted for publication February 2, 1995. ©AlphaMed Press 1066-5099/95/\$5.00/0

## Materials and Methods

### Source and Preparation of Cells

Bone marrow aspirates ( $n = 8$ ) were obtained from normal volunteers undergoing a general anesthetic for routine surgical procedures. Peripheral blood samples were obtained from patients with Hodgkin's disease ( $n = 2$ ), non-Hodgkin's lymphoma ( $n = 3$ ) or breast carcinoma ( $n = 3$ ), undergoing treatment with cyclophosphamide  $4 \text{ g/m}^2$  and rHuG-CSF  $5 \mu\text{g/kg}$  intravenously as previously described [17]. Peripheral blood samples were taken twice during the recovery phase following hypoplasia, as the peripheral blood leukocyte count rose above  $1 \times 10^9/\text{l}$ , and 48 h later. These time points correspond to the rise and subsequent fall in the levels of circulating progenitors in the peripheral blood [17]. These procedures were carried out with informed

consent and approved by the Lothian Health Board Ethics of Medical Research Subcommittee for Medicine and Clinical Oncology.

Samples were prepared by discontinuous density centrifugation over Ficoll Hypaque at  $1.077 \text{ g/ml}$  (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). Low buoyant-density cells from the interface were collected, and the viability was assessed by trypan blue dye exclusion (Sigma Chemical Co., Poole, UK). Cells were washed three times in a handling medium consisting of phosphate buffered saline containing 1% bovine serum albumin (Sigma) and 0.1% sodium azide (Sigma). In order to block Fc receptor uptake of the monoclonal antibody,  $5 \times 10^5$  cells/test were incubated with rabbit serum for 15 min, washed, then incubated for 30 min with  $10 \mu\text{l}$  of a pretitrated purified monoclonal antibody addressed to a cell adhesion molecule (Table I). After incubation, the

**Table I.** Cell adhesion molecules studied

Cell adhesion molecule (CD)	Ligand(s)	Monoclonal antibody (Source)	References
<b>Immunoglobulin Gene Superfamily</b>			
ICAM-1 (CD54)	LFA-1	84H10 (IT)	[6, 7]
PECAM-1 (CD31)	PECAM-1	5.6E (IT)	[8, 13]
	sulphated glycosaminoglycans		
LFA-3 (CD58)	LFA-2	AICD58 (IT)	[7]
<b>Integrin Family</b>			
<i><math>\beta_1</math> VLA subfamily</i>			
VLA-4 (CD49d/CD29)	VCAM-1	HP2/1 (IT)	[7, 9, 10, 12]
	fibronectin		
VLA-5 (CD49e/CD29)	fibronectin	SAM 1 (IT)	[7, 9, 10, 12]
<i><math>\beta_2</math> leukocyte adhesion subfamily</i>			
LFA-1 (CD11a/CD18)	ICAM-1, 2 & 3	IOT16 (IT)	[7, 9, 10-12]
<i><math>\beta_3</math> cytoadhesion subfamily</i>			
Vitronectin receptor (CD51/CD61)	vitronectin	AMF7 (IT)	[7]
<b>Selectin Family</b>			
L-Selectin (CD62L)	sialyl Lewis <sup>x</sup> CD34	Dreg56 (IT)	[13]
<b>Proteoglycan Analogues</b>			
HCAM(CD44)	collagen hyaluronic Acid	F10-44-2 (ST)	[7, 11, 14]
<b>CD36/LIMP II Family</b>			
(CD36)	collagen thrombospondin	FA6-152 (IT)	[15, 16]

Sources of monoclonal antibodies: Immunotech, Marseille, France (IT). Serotec, Oxford, UK (ST).



cells were washed three times and incubated for 30 min with a sheep antimouse IgG:R-phycoerythrin conjugate (Sigma) diluted to 1/40. The cells were washed three times and incubated for 15 min with mouse serum in order to block uptake of the anti-CD34 monoclonal antibody by the bound sheep anti-mouse reagent. The cells were washed again and incubated for 30 min with 10  $\mu$ l of anti-CD34 monoclonal antibody directly conjugated to fluorescein isothiocyanate (FITC) (8G12; Becton Dickinson Immunocytometry Systems, San Jose, CA). Finally the cells were washed and resuspended in 2% paraformaldehyde (Merck BDH, Poole, UK). Positive controls were established using purified and FITC-conjugated monoclonal antibodies addressed to CD45 (J33; IT). Negative controls were established using irrelevant isotype-specific purified and conjugated monoclonal antibodies as appropriate. All incubation, washing, centrifugation and storage were carried out at +4°C.

#### *Flow Cytometry*

Flow cytometric analysis was performed on a fluorescence-activated cell sorter (FACScan, Becton Dickinson) equipped with an argon laser tuned at 488 nm, power 14.8 mW. Compensation was set in the two fluorescence channels using the positive controls. Data acquisition was carried out with Consort 30 Data Management System. An initial acquisition on an ungated sample was used to define the distribution of the CD34<sup>+</sup> population on the forward and side scatter dot-plot and to establish a lymphoblastoid acquisition gate. This was found to reduce background fluorescence and enrich for HPC permitting better definition and less contamination of the CD34<sup>+</sup> population. Forward and side scatter and two fluorescence signals were determined for 10,000 events and stored in listmode data files. Analysis of the data was performed with Consort 30 software.

#### *Data Analysis*

Results are reported as mean  $\pm$  standard error of the mean of 8 replicate experiments. Statistical analyses were performed using a two-tailed Student's *t*-test for paired or unpaired samples as appropriate. *p* values were not formally corrected for the effects of multiple comparisons, but we have only recognized statistical significance at a *p* value of <0.01.

## **Results**

#### *Definition of the CD34<sup>+</sup> Population*

A marker established at 0.5% on the FL-1 histogram of the negative control was used to define the CD34<sup>+</sup> population. These comprised  $5.1 \pm 4.8\%$  of cells within the lymphoblastoid acquisition gate from bone marrow (*n* = 8). Adult peripheral blood taken at day  $10 \pm 1$  post-cyclophosphamide showed a white count of  $1.9 \pm 0.6 \times 10^9/l$ , and contained  $6.8 \pm 4.3\%$  CD34<sup>+</sup> cells (*n* = 7). Paired samples taken at day  $12 \pm 1$  showed a white count of  $16.7 \pm 3.1 \times 10^9/l$ , and contained  $5.3 \pm 5.3\%$  CD34<sup>+</sup> cells (*n* = 8).

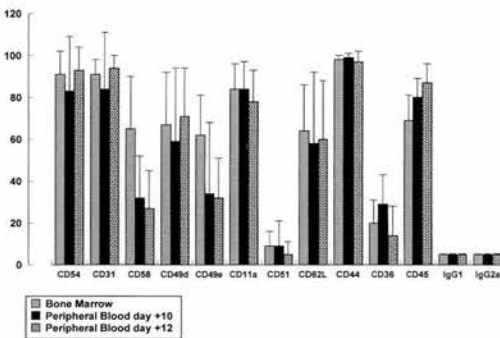
#### *Determination of Cell Adhesion Molecule Expression*

A marker established at the point of minimal inflection on the FL-2 histogram of the appropriate negative control was used to define adhesion molecule positivity. The mean fluorescence intensity of the CD34<sup>+</sup> population was calculated from the quadrant statistics. CD34<sup>+</sup> HPC expressed 9 out of the 10 cell adhesion molecules studied (Figs. 1-3). Virtually all bone marrow HPC expressed HCAM (CD44). ICAM-1 (CD54), PECAM-1 (CD31) and LFA-1 (CD11a) were also expressed by a high proportion of HPC, while intermediate to low percentages expressed LFA-3 (CD58), VLA-4 (CD49d), VLA-5 (CD49e), L-Selectin (CD62L) and CD36 (Fig. 1). When the mean fluorescence intensity of the CD34<sup>+</sup> population was analyzed, HCAM (CD44) displayed very high intensity fluorescence, while those of the other adhesion molecules were substantially lower (Fig. 2).

Peripheral blood HPC expressed less LFA-3 ( $p < 0.01$ ) and VLA-5 ( $p < 0.01$ ) than bone marrow HPC when compared by percentage expression, but not when compared by mean fluorescence intensity. There was no significant difference in cell adhesion molecule expression by paired peripheral blood HPC samples collected at two time points.

## **Discussion**

We have confirmed the observations of a number of other groups that bone marrow HPC express at least nine cell adhesion molecules belonging to several molecular families (Table I,

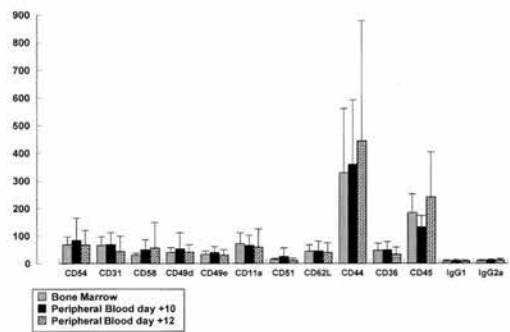


**Fig. 1.** Percentage cell adhesion molecule positivity within the CD34<sup>+</sup> population. Data are presented as the mean  $\pm$  standard error of eight replicate experiments, and CD45 and IgG1 are reported for comparison as positive and negative controls.

Figs. 1-3). In addition to these, CD34 may itself act as a ligand for L-Selectin [18] (CD62L), while CD45 and Thy-1 (CDw90) are known to mediate adhesion to heparan sulphate [19], and cell membrane-associated proteoglycans may mediate adhesion to fibronectin [20]. The way in which these various molecular pathways contribute to HPC homing and engraftment is complex and currently poorly understood.

The mechanisms responsible for HPC release from the marrow during migration and mobilization are similarly uncertain. We have investigated one hypothesis—that HPC egress may result from downregulation of cell adhesion molecule expression. Other possibilities include downregulation of HPC adhesion molecule function without alteration in expression, upregulation of adhesion molecules not normally expressed, or alteration in marrow endothelial function.

Mobilized peripheral blood HPC demonstrate less expression of LFA-3 (CD58) and VLA-5 (CD49e) than bone marrow HPC. The discordance between results calculated by percentage positivity and by mean fluorescence intensity may reflect the greater sensitivity of the former to loss of expression by a subpopulation of CD34<sup>+</sup> cells, as averse to a global reduction in expression by the CD34<sup>+</sup> population as a whole. LFA-3 is a counter-receptor for LFA-2, and may mediate regulatory interactions between T lymphocytes and HPC [21]. VLA-5 recognizes the cell binding domain of fibronectin, and modification of expression may contribute to the

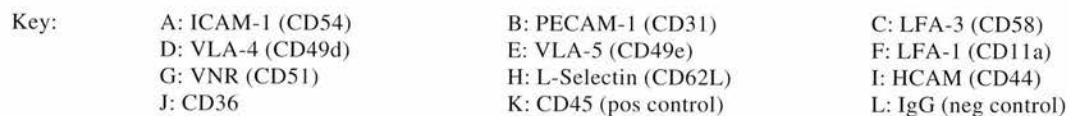


**Fig. 2.** Mean fluorescence intensity of the CD34<sup>+</sup> population. Data are presented as the mean  $\pm$  standard error of eight replicate experiments. CD45 and IgG1 are reported for comparison as positive and negative controls.

observed lineage and stage-specific binding of HPC to fibronectin [22, 23], and to the release of cells from the marrow.

The overall physiological relevance of this data remains unclear for two principal reasons: 1) differential adhesion molecule expression between HPC subsets [24] and source-dependent differences in the heterogeneity of the CD34<sup>+</sup> population [25, 26] may contribute to the observed differences in adhesion molecule expression by the CD34<sup>+</sup> population as a whole and 2) the functional role of various cell adhesion molecule—ligand pathways in HPC—stromal interaction remains to be fully established, and it is unclear whether observed phenotypic differences are reflected by functional differences in the adhesion behavior of sessile and circulating HPC.

Finally, we considered the possibility that differences in cell adhesion molecule expression may be detectable between circulating HPC during the early phase of mobilization, when marrow egression predominates, and the later phase, when HPC may be returning to the marrow. We demonstrated no such differences and offer two possible explanations. Return of HPC cell adhesion molecule expression to “normal” may lead to rapid clearance of these cells from the circulating population, or levels of circulating HPC may fall through apoptosis of nonengrafted cells rather than through return to the marrow. The fate of mobilized HPC clearly requires further investigation.



## Acknowledgments

Our thanks to *Drs. Jenny Craig, James Garden and Robert Leonard* for help in the provision of samples and *Dr. Michael Mackie* for support of the flow cytometry. Critical appraisal was provided by *Drs. John Ansell and Robin Prescott*. This research was supported by the Melville Trust for the Care and Cure of Cancer, the Kay Kendall Leukaemia Trust and the Peel Medical Research Trust.

## References

- 1 Tavassoli M. Embryonic and fetal hemopoiesis: an overview. *Blood Cells* 1991;17:269-281.
- 2 Tavassoli M, Hardy C. Molecular basis of homing of intravenously transplanted stem cells to the marrow. *Blood* 1990;76:1059-1070.
- 3 Craig JIO, Turner ML, Parker AC. Peripheral blood stem cell transplantation. *Blood Rev* 1992;6:59-67.
- 4 Broxmeyer HE. Self-renewal and migration of stem cells during embryonic and fetal hematopoiesis: important but poorly understood events. *Blood Cells* 1991;17:282-286.
- 5 Turner ML. Regulation of hematopoietic progenitor cell migration, mobilization and homing. *STEM CELLS* 1994;12:227-229.
- 6 Arkin S, Naprstek B, Guarini L et al. Expression of intracellular molecule-1 (CD54) on hematopoietic progenitors. *Blood* 1991;77:948-953.
- 7 Saeland S, Duvert V, Caux C et al. Distribution of surface-membrane molecules on bone marrow and cord blood CD34<sup>+</sup> hematopoietic cells. *Exp Hematol* 1992;20:24-33.
- 8 Waft SM, Williamson J, Genevier H et al. The heparin binding PECAM-1 adhesion molecule is expressed by CD34 positive hematopoietic precursor cells with early myeloid and B lymphoid cell phenotypes. *Blood* 1993;82:2649-2663.
- 9 Soligo D, Schiró R, Luksch R et al. Expression of integrins in human bone marrow. *Br J Haematol* 1990;76:323-332.
- 10 Liesveld JL, Winslow JM, Frediani KE et al. Expression of integrins and examination of their adhesive function in normal and leukemic hematopoietic cells. *Blood* 1993;81:112-121.
- 11 Kansas GS, Muirhead J, Dailey MO. Expression of the CD11/CD18 leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans. *Blood* 1990;76:2483-2492.
- 12 Papayannopoulou T, Brice M. Integrin expression profiles during erythroid differentiation. *Blood* 1992;79:1686-1694.
- 13 Simmons PJ, Niutta S, Ashman LK et al. Primitive human bone marrow progenitors express L-Selectin and PECAM-1 (CD31). *Exp Hematol* 1992;20:829a.
- 14 Lewinsohn DM, Nagler A, Ginzton N et al. Hemopoietic progenitor cell expression of the H-CAM (CD44) homing-associated adhesion molecule. *Blood* 1990;75:589-595.
- 15 Long MW, Dixit VM. Thrombospondin functions as a cytoadhesion molecule for human hematopoietic progenitor cells. *Blood* 1990;5:2311-2318.
- 16 Greenwalt JS, Lipsky RH, Ockenhouse CF et al. Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. *Blood* 1992;80:1105-1115.
- 17 Craig JIO, Anthony RS, Stewart A et al. Peripheral blood stem cell mobilization using high dose cyclophosphamide and G-CSF in pre-treated patients with lymphoma. *Br J Haematol* 1993;85:210-212.
- 18 Baumhueter S, Singer MS, Henzel W et al. Binding of L-Selectin to the vascular sialomucin CD34. *Science* 1993;262:436-438.
- 19 Parish CR, Hogarth PM, McKenzie IF. Evidence that Thy-1 and Ly-5 (T-200) antigens interact with sulphated carbohydrates. *Immunol Cell Biol* 1988;66:221-230.
- 20 Minguell JJ, Hardy CL, Tavassoli M. Membrane-associated chondroitin sulphate proteoglycan and fibronectin mediate the binding of hemopoietic progenitor cells to stromal cells. *Exp Cell Res* 1992;201:200-207.
- 21 Pantel K, Nakeff A. The role of lymphoid cells in hematopoietic regulation. *Exp Hematol* 1993;21:738-742.
- 22 Tsai S, Patel V, Beaumont E et al. Differential binding of erythroid and myeloid progenitors to fibroblasts and fibronectin. *Blood* 1987;69:1587-1594.
- 23 Ballard LL, Brown EJ, Holers VM. Expression of the fibronectin receptor VLA-5 is regulated during human B cell differentiation and activation. *Clin Exp Immunol* 1991;84:336-346.
- 24 Gunji Y, Nakamura M, Hagiwara T et al. Expression and function of adhesion molecules on human hematopoietic stem cells: CD34<sup>+</sup> LFA-1<sup>+</sup> cells are more primitive than CD34<sup>+</sup> LFA-1<sup>-</sup> cells. *Blood* 1992;80:429-436.
- 25 Bender JG, Unverzagt KL, Walker DE et al. Identification and comparison of CD34 positive cells and their subpopulations from normal peripheral blood and bone marrow using multi-color flow cytometry. *Blood* 1991;77:2591-2596.
- 26 Bender JG, Williams SF, Myers S et al. Characterization of chemotherapy mobilized peripheral blood progenitor cells for use in autologous stem cell transplantation. *Bone Marrow Transplant* 1992;10:281-285.

## **ACKNOWLEDGEMENTS.**

My thanks are due to the many people without whom this work would not have been possible. I am indebted to my supervisors Dr John Ansell and Dr Alistair Parker for their guidance and support. My thanks to Dr Willy Murphy, Dr John Sweetenham and the numerous friends, colleagues and patients from the Department of Haematology Royal Infirmary of Edinburgh, the Institute of Cell Animal and Population Biology University of Edinburgh, Radiopharmacy Royal Infirmary of Edinburgh, Edinburgh and SE Scotland Blood Transfusion Service, and the CRC Medical Oncology Unit, University of Southampton. Financial support was provided by the Melville Trust for the Care and Cure of Cancer, the Kay Kendall Leukaemia Trust, the Leukaemia and Bone Marrow Transplant Fund, the Peel Medical Research Trust, Tenovus-Scotland, the Leukaemia Research Fund and Edinburgh and SE Scotland Blood Transfusion Service. Finally, my thanks are due to my wife and family for their encouragement and patience.